Endemic and Emerging Diseases of British Garden Birds

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ABSTRACT

In order to gain a robust understanding of the endemic and emerging diseases of wildlife populations, comprehensive and long-term disease surveillance is required. The aim of this thesis was to investigate causes of garden bird mortality across Great Britain utilising a novel technique combining two independent, yet complementary, surveillance schemes. Opportunistic reports of garden bird mortality were solicited from the general public and co-ordinated through national reporting lines and the RSPB Wildlife Enquiries Unit. A major limitation of such *ad hoc* reporting schemes is their vulnerability to spatial, temporal and other biases. Structured, systematic surveillance was achieved through collaboration with the British Trust for Ornithology's Garden Bird Watch scheme; c. 750 participants were recruited from across Great Britain. This citizen science network provided information on a weekly basis on bird morbidity and mortality in their gardens, including all species and suspected causes of death. In addition, information was supplied on the species and numbers of birds visiting the garden along with details of provisioning and hygiene practices at bird feeding stations. This unique systematic dataset enabled the findings of the larger opportunistic scheme to be corroborated. A network of disease investigation centres performed *post mortem* examinations on birds submitted following standardised protocols. In excess of 1,500 post mortem examinations, on a total of 49 species from 23 families, were performed between 1st April 2005 and 31st March 2008 with comprehensive geographical coverage across Great Britain. Cause of death categories were found to vary significantly between avian families with infectious disease diagnosed most frequently in gregarious, granivorous species. Dramatic changes occurred in the epidemiology of some of the infectious diseases considered well-known in British garden birds over the short study period. A sharp decline occurred in the number of mortality incidents caused by salmonellosis, an endemic bacterial disease, coupled with a shift in the dominant *S.* Typhimurium phage type from DT40 to DT56(v). Trichomonosis, an endemic parasitic disease of native columbiforms, emerged in Fringillidae species in 2005. Epidemic seasonal mortality occurred in 2006 and 2007 due to trichomonosis, leading to a significant population decline of the greenfinch and chaffinch that matched the epidemic in time and space. Avian pox infection, an endemic viral pathogen, emerged in Paridae species, causing florid lesions and localised outbreaks of disease. Evidence of mycotoxin exposure to British garden birds was found and supplementary bird foods were confirmed as a potential source; the pathological significance of this exposure and relative importance of supplementary food as a source of toxins remains as yet unknown. This study adopted a conservation medicine approach and benefited from collaboration with a multi-disciplinary team and the general public. This study offers a successful and financially viable model that has the potential to be expanded to incorporate health surveillance of other positively-perceived wildlife species within garden habitats in the future.

PREFACE

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LIST OF ABBREVIATIONS

ACAF	Advisory Committee on Animal Feedingstuffs
AF	Aflatoxin
API	Analytical Profile Index
BAP	Biodiversity Action Plan
bp	base pair
BLAST	Basic Local Alignment Search Tool
BBS	Breeding Bird Survey
BTO	British Trust for Ornithology
BW: MTL	Body weight/maximum tarsus length ratio
CSIP	Cetacean Strandings Investigation Programme
COD	Cause of death
df	Degree(s) of freedom
DT	Definitive phage type
EID	Emerging infectious disease
EU	European Union
FSA	Food Standards Agency
GBHi	Garden Bird Health *initiative*
GBW	Garden BirdWatch
GIS	Geographical Information Systems
GOR	Government office region
HPA	Health Protection Agency
HPAI	Highly pathogenic avian influenza
HPLC	High pressure liquid chromatography
ITS	Internal Transcribed Spacer
IoZ	Institute of Zoology
MPL	Maximum permissible level
NCBI	National Centre for Biotechnology Information
OA	Ochratoxin A
OPD	Oligonucleotide Probe Database
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PM: MTL	Superficial pectoral muscle mass/maximum tarsus length ratio
PME	Post mortem examination
PSG	Phosphate Saline Glucose
RDNC	Reacts Does Not Conform
RSPB	Royal Society for the Protection of Birds
RAPD	Random Amplified Polymorphic DNA
SRU	Salmonella Reference Unit
TRS	Tick-Related Syndrome
UV	Ultra violet
VLA	Veterinary Laboratories Agency
XLD	Xylose-Lysine Deoxycholate
ZSL	Zoological Society of London
UKAS	United Kingdom Accreditation Scheme

MAIN CONTENTS OF THESIS

CHAPTER 1: INTRODUCTION - CURRENT UNDERSTANDING OF THE INFECTIOUS DISEASES OF GARDEN BIRDS IN GREAT BRITAIN

Endemic disease is defined as either '*the usual frequency of occurrence*' or '*the constant presence*' of a disease in a population (Thrusfield 1995). Epidemic disease is defined where the level of a disease exceeds that of the anticipated endemic level (Thrusfield 1995). Emerging infectious diseases (EIDs) are defined as '*diseases that have recently increased in incidence or geographic range, recently moved into new host populations, recently been discovered or are caused by newly-evolved pathogens*' (Lederberg et al., 1992; Morse 1993; Daszak et al., 2000; Daszak et al., 2001). Effective surveillance is essential to develop an understanding of endemic pathogens within wildlife populations, enabling identification of epidemic and EIDs when they occur (Sainsbury et al., 2001).

There have been no recent comprehensive surveys of the infectious or non-infectious diseases of garden bird species' in Great Britain. Jennings (1954) reviewed the findings of post mortem examination from 112 wild birds, including passerine species, and concluded that a variety of pathogens had contributed to the cause of death of some individuals including mycobacteriosis and avian pox infection in the wood pigeon (*Columba palumbus*), and *Pasteurella multocida* infection in a starling (*Sturnus vulgaris*). Keymer (1958) performed post mortem examinations on 513 British birds from 74 species over a 3-year period which included submissions from feral, semi-domesticated and captive environments. He concluded that 'natural hazards' (such as starvation), traumatic injury (collision and predation), toxins and infectious disease were frequent causes of death for the feral bird submissions. He diagnosed infection with a range of bacterial (e.g. *Yersinia pseudotuberculosis*, *Pasteurella multocida*), fungal (e.g. *Aspergillus fumigatus*) and viral (e.g. avian pox) pathogens in passerine species. Baker (1977) examined 132 birds of 33 species collected from the Wirral region of Cheshire and found a similar range of pathogens present to the previous studies.

Pennycott et al. (1998a) reviewed the causes of mortality within Fringillidae species from Scotland and found infectious disease to be a common cause of death, chiefly

salmonellosis and colibacillosis due to *Escherichia coli* serotype O86. More recent publications have focused on specific pathogens, principally *Salmonella enterica* subspecies 1. serovar Typhimurium (Le Minor et al., 1997) definitive phage type (DT)40 and DT56 variant (v) (Pennycott et al., 2006).

Out with the peer-reviewed literature, case reports and summaries of wild bird disease incidents are produced by the Veterinary Laboratories Agency (VLA) as part of their Diseases of Wildlife Scheme which was launched in 1998. Contributions from multiple research centres, universities, government-funded institutions, independent scientists and wildlife rehabilitation centres involved with wildlife disease investigation across the UK are collated and this information forms the basis of the annual report submitted to the OIE – World Organisation for Animal Health. The VLA outputs report a similar range of pathogens affecting passerine populations as in the peer-reviewed literature (VLA 2008).

In 2005, at the outset of this research, examples of infectious disease that were considered endemic within wild bird populations included salmonellosis in greenfinches (*Carduelis chloris*) and house sparrows (*Passer domesticus*) (Pennycott et al., 2006), colibacillosis in Fringillidae species (greenfinches and siskins (*Carduelis spinus*)) (Pennycott et al., 2002), trichomonosis in Columbidae (Cousquer 2003), and avian pox infection in dunnocks (*Prunella modularis*), house sparrows and starlings (Pennycott 2003). Localised disease outbreaks, typically restricted to individual garden habitats and their locality, have been described due to salmonellosis and colibacillosis, but there were no known large-scale epidemics of wild bird disease in Great Britain in spring 2005. *Suttonella ornithocola* was first described in association with Paridae species mortality in 2006, and so could be considered an EID of tit species based on its recent discovery; however, the tit mortality incidents from which this novel bacterium was isolated occurred in 1996.

In this chapter, aspects of the literature on important bacterial (i.e. Salmonella *Typhimurium*), viral (avian pox) and parasitic (*Trichomonas gallinae*) pathogens of British garden birds relevant to the focus in this thesis are introduced, and a group of toxins to which these avian populations may be exposed (mycotoxins).

BACTERIAL

i. *Salmonella* Typhimurium.

Garden bird salmonellosis

Studies of passerine bird mortality have identified salmonellosis as a significant, often the most important, infectious cause of death (Refsum et al., 2003; Hall et al., 2008). Salmonellosis outbreaks affecting small passerine species have been documented since at least the mid 20th Century, with early reports from the UK (Wilson et al., 1967; Macdonald et al., 1969; Macdonald et al., 1974), continental Europe (Schaal et al., 1967; Hurvell et al., 1974; Nielsen et al., 1975), Canada (Wobeser et al., 1969) and the U.S.A. (Hudson et al., 1957; Faddoul et al., 1966). Some authors, however, consider salmonellosis to be an EID of wild birds since its prevalence appears to have increased over the past 40 years (Tizard 2004), particularly in association with anthropogenic provisioning (Kirkwood et al., 1995; Tizard 2004). The bacterium, *S.* Typhimurium is the causative agent of these incidents. Sporadic outbreaks of disease are most commonly associated with garden feeding stations, although the potential for large-scale mortality beyond localised sites exists, as evidenced by epidemic losses in Canada, the U.S.A., New Zealand and Japan (Friend et al., 1999a; Alley et al., 2002; Une et al., 2008).

Globally, granivorous passerine birds are those most commonly reported to be affected by salmonellosis. In the U.S.A. and Canada, the pine siskin (*Carduelis pinus*), American goldfinch (*Carduelis tristis*), northern cardinal (*Cardinalis cardinalis*), evening grosbeak (*Hesperiphona vespertina)* and the common redpoll (*Carduelis flammea*) (Locke et al., 1973; Prescott et al., 1998; Daoust et al., 2000; Hall et al., 2008) are the species primarily involved in salmonellosis mortality incidents, whereas the most commonly-affected species in Norway is the bullfinch (*Pyrrhula pyrrhula*) (Refsum et al., 2002a) and, in New Zealand, the (introduced) house sparrow (Alley et al., 2002). In Great Britain, the greenfinch and the house sparrow are the species most frequently reported

with the disease (Kirkwood et al., 1998; Pennycott et al., 2006). The majority of research on wild bird *Salmonella* infection has been undertaken in Scotland (Macdonald et al., 1974; Pennycott et al., 1998a). A comprehensive survey of salmonellosis in native garden bird species across Great Britain is required to further understand the epidemiology of the disease, the species affected and to identify any spatial or temporal trends that occur.

Salmonella spp. carriage in passerine populations

Survey results for *Salmonella* spp. carriage in passerine populations vary with the habitat type in which the birds were sampled. For example, the prevalence of infection in farmland habitats may by greater where sympatric livestock infection occurs; the results of farmland studies may not accurately reflect rates of *Salmonella* spp. carriage in normal passerine populations (Tizard 2004).

Surveys of migratory birds in Sweden using a combination of faecal samples and cloacal swabs found minimal evidence of *Salmonella* spp. infection. Passerines (n=101) on their return from migration were screened at 3 ringing sites and all were found to be negative for *Salmonella* spp. infection although the species were principally thrushes (*Turdus* spp.) and starlings and did not include any finches or house sparrows (Palmgren et al., 1997). In a separate study in Sweden, a single isolate of *Salmonella schleissheim* was identified from a mistle thrush (*Turdus viscivorous*) from 2377 screened wild birds from 110 species (Hernandez et al., 2003).

Morishita et al. (1999) screened 1709 passerines in Ohio at 38 field sites using cloacal swabs and found infection in 4/373 house sparrows (1%) and no infection in the finches (416 house finch (*Carpodacus mexicanus*), 11 American goldfinch and 1 purple finch (*Carpodacus purpureus*)) examined. Brittingham et al. (1988) screened cloacal swabs collected from 364 healthy passerines and woodpeckers in rural habitats of mixed deciduous woodland; 3 of the 5 sites incorporated garden feeding stations. All birds were negative for *Salmonella* spp. infection, although the sample comprised predominantly of black-capped chickadees (*Parus atricapillus*) which are not a species

in which salmonellosis has been frequently reported; finch species accounted for only 9% (32/364) of the birds examined.

Refsum et al. (2003) screened 1990 small passerines of 7 species caught at ringing sites with feeding stations in Norway for *Salmonella* spp. using cloacal swabs and found only 2% of birds to be positive on culture. Infection was confirmed in the species in which clinical disease is typically observed i.e. the bullfinch, greenfinch, common redpoll, siskin and house sparrow.

Research is required to examine the prevalence of asymptomatic infection with *Salmonella* spp. in garden bird species in Great Britain and to determine, when this occurs, whether the serotype and phage types match those responsible for clinical disease and whether the species are those typically affected by the disease.

Passerines as a primary reservoir of infection for host-adapted *S.* Typhimurium phage types

S. Typhimurium DT40, DT56(v) and DT160 account for the majority of isolates reported from garden birds in Great Britain (Penfold et al., 1979; Macdonald et al., 1980; Pennycott et al., 2006). Polymerase chain reaction (PCR) virulotyping identified virulence-associated genes associated with intra-cellular survival, cell adhesion and invasiveness in these wild bird phage types indicating that they may be capable of systemic and enteric salmonellosis in avian and mammalian hosts (Hughes et al., 2008). In addition, the strains were able to invade and persist in avian macrophages, indicating their ability to cause systemic disease in birds (Hughes et al., 2008).

Garden bird *S.* Typhimurium phage types, particularly DT40, are thought to have a narrow host range and may be highly host-adapted unlike other serotype Typhimurium variants with broad host-ranges such as DT104 (Rabsch et al., 2002). Pulsed-field gel electrophoresis (PFGE) studies indicate that garden bird *S.* Typhimurium phage types are clonal in some instances (e.g. DT160) (Alley et al., 2002). Refsum et al. (2002) found that all 16 *S.* Typhimurium DT40 isolates within their study clustered in the same

pulsed-field group and that the patterns for passerine isolates were consistent over their study period of 20 to 30 years. These findings support the hypothesis that wild passerine populations act as the primary reservoir of these *S.* Typhimurium phage types. Research is required to examine the *S.* Typhimurium phage types that currently affect passerine populations in Great Britain, and to assess the evidence for clonality or variation in their PFGE profiles. This biotyping data could be used to examine annual and spatial trends in passerine salmonellosis for the first time across Great Britain.

Experimental infection of house sparrows with *S.* Typhimurium DT160 indicated that this species can act as a persistent carrier excreting bacteria intermittently, and in a dose-dependent manner, for a period of at least 10 days following infection (Connolly et al., 2006). Consequently garden bird species may act as a source of infection through faecal contamination, for example at garden bird feeding stations. Microbiological monitoring of wild bird faeces collected at garden bird feeding stations and from finches caught using mist nets has confirmed that sites with a history of garden bird salmonellosis may have a relatively high proportion of contaminated samples, particularly during the winter months (Pennycott et al. 2002, 2006; Grant et al., 2007).

Genotypically identical strains of *S.* Typhimurium have been found in sympatric populations of wild birds, cats and humans (Hauser et al., 2009) indicating that spill-over may occur to companion animals, humans, and potentially to livestock and other wildlife species.

Epidemiology of passerine salmonellosis and spill-over of infection to other taxa

Pennycott et al. (2006) classified 'wild bird' strains of *S.* Typhimurium (including DT40, DT56(v) from small passerines, DT41 and DT195 principally from Laridae; and DT2 and DT99 frequently isolated from Columbidae) and found that these strains were infrequently isolated from livestock: they accounted for less than 0.5% of the isolates recovered from cattle, sheep, pigs, chickens and turkeys between 1995 and 2003 in Great Britain. The 'wild bird' strains of *S.* Typhimurium were more frequently isolated from reared birds under extensive management systems including game birds, ducks and

geese, where increased frequency of contact with free-living birds would be predicted, however, they still only accounted for around 3% of isolates overall (Pennycott et al., 2006). These findings indicate that wild birds are not an important source of salmonella infection in livestock and poultry in Great Britain although the potential for transmission does occur. Skov et al. (2008) found evidence in Denmark that *Salmonella* bacteria were transmitted from production animals to wildlife species, rather than vice versa, and that this occurred principally during outbreaks of salmonellosis in livestock.

Sick garden birds suffering with salmonellosis may be predisposed to predation by companion animal and wildlife predators. Clinical disease in cats due to garden bird *S.* Typhimurium phage types has been termed 'songbird fever' (Scott 1988). PFGE identified identical patterns in *S.* Typhimurium isolates from small passerines and cats in Norway supporting the epidemiological link (Refsum et al., 2002b). An outbreak of feline salmonellosis associated with anorexia, pyrexia, vomiting and diarrhoea occurred in Sweden in 1999; some of the affected pets had a history of feeding on wild birds (Tauni et al., 2000). Salmonellosis was seen in domestic cats with a history of preying on sick birds in the winter of 1992-1993 in Canada, contemporaneous with a period of epidemic passerine mortality due to *S.* Typhimurium DT40 (Daoust et al., 2000). Philbey et al. (2009) reviewed cases of salmonellosis in domestic cats that occurred in the UK between 1955 and 2007 and found that 14% (14/100) of cases were caused by *S.* Typhimurium DT40 and DT56(v). Nine cats with enteric disease caused by the avian strains of *S.* Typhimurium in the UK all had a history of hunting wild birds (Philbey et al., 2008). Clinical signs included pyrexia, anorexia and diarrhoea or dysentery for variable periods of up to 3-weeks duration. The majority of cases presented between November and February, mirroring the months when salmonellosis in small passerine species most commonly occurs in the UK (Philbey et al., 2008).

Whilst garden bird *S.* Typhimurium isolates are infrequently isolated from horses the potential for infection exists. Macdonald et al. (1980) reported a number of equine salmonellosis cases in the south of England between 1996 and 1972 of the same phage types affecting wild passerines. Also *S.* Typhimurium DT160 infected horses in New

Zealand during outbreaks of the same bacterial infection in wild birds in 2000 (Alley et al., 2002). In Great Britain, 'wild bird strain' *S.* Typhimurium isolates were recovered from 13 horses between 1990 and 1996 (Pennycott et al., 1998a). Faecal contamination of stable blocks by peri-domestic species, such as the house sparrow, represents a likely source of infection in these cases (Tizard 2004).

Free-living wild animals (n=5494), including 16 mammal species (badgers *Meles meles* (n=4881), red fox (*Vulpes vulpes*) (n=188), grey squirrel (*Sciurius carolinensis*) (n=37), mole (*Talpa europea*) (n=34), field mouse (*Apodemus sylvaticus*) (n=3), brown rat (*Rattus norvegicus*) (n=23) and others), were screened for *Salmonella* spp. carriage in Cornwall over the period 1974 to 1988 (Euden 1990). The only mammal species from which *S.* Typhimurium was isolated was the badger (0.7% infected, 34/4881 individuals) and no garden bird phage types (DT40, DT56(v) or DT160) were found (Euden 1990). A more recent study of badgers in the north of England found evidence of infection with non-typhoidal salmonellae but no cases of *S.* Typhimurium (Wilson et al., 2003).

The red fox is the wild mammal reported to have the most frequent carriage of *Salmonella* spp in Sweden. Three percent (n=12) of foxes examined from 1972 to 1979 were intestinal carriers of *Salmonella* spp. of which 8 were *S.* Typhimurium (Mörner et al., 1993). A Norwegian study screened shot red fox carcasses for evidence of *Salmonella* spp. enteric carriage and most frequently isolated *S.* Typhimurium 4,12:i:1,2 with an identical PFGE profile to that from sympatric small passerines (Handeland et al., 2008). The peak in isolates matched the seasonality of disease outbreaks in Norwegian small passerines supporting the assumption that red fox infection occurred subsequent to predation and ingestion of small passerine prey. Experimental infection of silver foxes (*Vulpes vulpes*) with the *S.* Typhimurium 4,12:i:1,2 strain led to sub-clinical intestinal infection for 2 weeks, indicating that foxes may play in a role in the epidemiology of the bacterium (Handeland et al., 2008).

It has been suggested that rodent species attracted to garden bird feeding stations may act as a source of infection for people or small passerines, or as a vector of infection to

other species (Wilson et al., 1967). A study to evaluate faecal contamination of livestock food by rodents and other wildlife species on farms identified that this route should be considered as a risk of disease transmission to livestock (Daniels et al., 2003); a similar route could occur for birds visiting garden bird feeding stations contaminated with rodent faeces. However, longitudinal studies of wild rodent populations found minimal evidence of *Salmonella* spp. infection and concluded that they do not constitute a significant reservoir of infection for other species (Jones et al., 1976; Pocock et al., 2001). Where *Salmonella* spp. infection in wild rodents is found, studies have concluded that they are likely to become infected following contact with infected livestock (Robinson et al., 1968; Jones et al., 1976). Anecdotal evidence exists where a sample of wild brown rat faeces was positive for *S.* Typhimurium DT40 in a garden with contemporaneous small passerine mortality due to salmonellosis (Pennycott et al., 2002). Comprehensive studies monitoring enteric carriage of peri-domestic wild rodents, particularly house mice (*Mus musculus*) and brown rats, in the vicinity of garden bird feeding stations, are required to determine the prevalence of infection with *S.* Typhimurium and the role that rodents may play in the ecology of garden bird salmonellosis.

S. Typhimurium DT160 was confirmed to be the pathogen responsible for an outbreak of gastroenteritis in humans in a hospital in England; house sparrows were considered to be the source of infection through faecal contamination of the kitchen facilities (Penfold et al., 1979). Where epidemic mortality due to salmonellosis has occurred in garden birds, examples exist where large numbers of humans have also become infected and clinically affected. In Sweden in late February 1999 there was an outbreak of *S.* Typhimurium DT40 infection in passerines, cats and people. During the first 6 months of the year, 57 of the 242 domestically acquired human cases of salmonellosis were from strains found in wild birds and cats (Tauni et al., 2000). *S.* Typhimurium DT160, a previously unrecognized phage type in New Zealand, caused outbreaks of mortality in house sparrows in New Zealand in 2000 and contemporaneous disease in humans (Alley et al., 2002).

Children, the elderly and the immunocompromised generally are the most susceptible to severe illness due to salmonella infection (Kirkwood et al., 1997). Whilst *Salmonella* spp. infection in garden birds is recognised as potentially zoonotic, there has been no formal assessment of the epidemiological link between human and garden bird infection in Great Britain. Exploration of annual, spatial and temporal links between garden bird salmonellosis and human cases of *S.* Typhimurium infection with matched phage types would facilitate this assessment.

ii. *Escherichia coli* serotype O86.

Escherichia coli serotype O86:K61 was first confirmed as a cause of finch mortality in the Highland region of Scotland in 1994 and 1995, where the disease accounted for 87% and 93% of cases examined in each year respectively (Foster et al., 1998; Pennycott et al., 1998a). *E. coli* O86 infection was confirmed in chaffinches (*Fringilla coelebs*), greenfinches and siskins; however, no cases were observed in 101 non-finch species examined post mortem (Pennycott et al., 1998a). Affected birds were typically in good to moderate body condition. Many of the birds had food contents in the upper alimentary tract, some with large volumes of ingesta, whilst the gizzard usually contained grit with scant contents. The intestinal tract was either empty or contained discoloured fluid contents (Pennycott et al., 1998a). No other gross abnormalities were described on post mortem examination and histopathological examination of the alimentary tract was limited due to autolysis (Foster et al., 1998; Pennycott et al., 1998a).

E. coli O86 was isolated from all small intestinal samples, and most liver samples, that were examined from affected birds. The isolates were characteristically late lactose fermenting or non-lactose fermenting with analytical profile index (API) 20E profiles of either 4144102 or 5144102 (with the profiles differing due to the β-galactosidae reaction only) (Foster et al., 1998). PCR virulotyping studies confirmed that the *E. coli* O86 isolates are positive for cytolethal distending toxin, characteristic of enteropathogenic *E. coli*, and for the *eaeA* gene coding gamma-like intimin responsible for adhesion of attaching and effacing *E. coli* during infection; they are negative for verotoxin gene sequences (VT1 and VT2) and shiga-like toxins (Foster et al., 1998; La Ragione et al.,

2002). Experimental studies with *E. coli* O86 *in vitro* showed that the bacteria attached and invaded HEp-2 cells successfully (La Ragione et al., 2002). *In vivo* infection of domestic fowl chicks resulted in colonnisation, persistence and invasion of the intestinal tract although minimal pathology and no mortality occurred (La Ragione et al., 2002). However the authors noted that host factors may influence the outcome of infection and that finch species may be more susceptible to the infection (La Ragione et al., 2002).

E. coli O86 infection has been reported in Poland as a cause of nestling mortality in Passeridae species, including house sparrows and tree sparrows (*Passer montanus*) (Pawiak et al., 1991) and has been recovered from pheasants in Scotland (Foster et al., 1998). The bacteria have been reported as a cause of cellulitis in broiler chickens (Peighambari et al., 1995) but had not been isolated from commerical poultry in the UK before 2002 (R. Davies, *pers. comm.* cited by La Ragione et al., 2002). Mammalian infection has been described as a cause of diarrhoea in calves and pigs (Sojka 1965; Blanco et al., 1993; Alexa et al., 1995). *E. coli* O86 infection can cause infantile diarrhoea in humans (Ewing 1986) and the bacterium has been isolated sporadically from people in Great Britain over recent years (D. Munro and T. Cheasty, *pers. comm.* cited by Foster et al., 1998). Given the virulence factors possessed by the finch *E. coli* O86 isolates, Foster et al. (1998) concluded that this wild bird infection should be considered potentially zoonotic.

Microbiological examination of bird faeces collected from 2 sites with garden bird feeding stations in Scotland found no samples positive for *E. coli* O86, although the bacteria were isolated from bird carcasses submitted from the site during the period of monitoring. The reason why no *E. coli* O86 isolates were recovered may be due to the lack of sensitive and selective media used in the microbiology protocol, or may relate to the species visiting the sites since relatively few siskins and greenfinches were observed (Pennycott et al., 2002, 2006).

No comprehensive study has been performed to assess the incidence and epidemiology of colibacillosis in garden birds due to *E. coli* O86 infection across Great Britain.

Research is required to investigate the species affected, seasonality, distribution, and lesions observed due to *E. coli* O86 infection in garden birds across Great Britain.

iii. *Suttonella ornithocola*

Kirkwood et al. (2006) described their investigations into the morbidity and mortality of Paridae and Aegithalidae species at 11 disparate gardens in the spring of 1996; the species comprised the blue tit (*Cyanistes caeruleus*), coal tit (*Periparus ater*), great tit (*Parus major*) and long-tailed tit (*Aegithalos caudatus*). In total 34 dead birds were reported, ranging from 1–10 per incident. Post mortem examinations revealed pulmonary congestion but no other significant abnormalities. *Suttonella ornithocola* was isolated from the lungs of affected birds (Kirkwood et al., 2006) and was identified as a novel bacterium belonging to the family *Cardiobacteriaceae* (Foster et al., 2005). Kirkwood et al. (2006) postulated that *S. ornithocola* infection might have caused the deaths of these birds, but could reach no firm conclusion as to the association between *S. ornithocola* and disease or mortality since no tissues were available for histopathological examination.

PARASITIC

i. *Trichomonas gallinae*

Species affected by trichomonosis

Trichomonas gallinae, first discovered by Rivolta (1878) and named by Stabler (1938), is an amitochondrial anaerobic protist in the family Trichomonadidae (phylum Parabasalia order Trichomonadida). The rock pigeon (*Columbia livia*), an Old World species, is considered to be the reservoir host of this parasite (Stabler 1954). *T. gallinae* principally infects columbid species, and birds of prey (Accipitriformes (Real et al., 2000) and Strigiformes (Pokras et al., 1993)) that feed on sick pigeons and doves, although other avian species are less commonly affected (Forrester et al., 2008; Anderson et al., 2009). A range of Anseriformes, Charadriiformes, Galliformes, Gruiformes, Psittaciformes and Struthioniformes species have been shown to be susceptible hosts for *T. gallinae* when held in captivity and when used in experimental infection studies (Forrester et al., 2008; Bunbury 2006). For example, trichomonosis is

known to be a particular clinical problem in captive budgerigars (*Melopsittacus undulatus*) where the disease is characterised by signs of vomition (Baker 1986).

Trichomonosis in passeriform species has been less frequently reported. Experimental studies have shown susceptibility to disease in the Java sparrow (*Padda oryzivora*) (Callender et al., 1937), canary (*Serinus canaria*) and house sparrow (Levine et al., 1941). Disease caused by *T. gallinae* has been described in captive finches in zoological collections and domestic aviaries (Sandmeier et al., 2006). In Alberta, mortality due to an outbreak of trichomonosis occurred in a mixed finch species exhibit in a private aviary (Bengalese finch (*Lonchura striata* var *domestica*), golden-breasted finch (*Amandava subflava*), blue-faced parrot finch (*Erythrura trichora*)) where severe respiratory distress was observed, and mortality, associated with upper alimentary tract lesions from which a *Trichomonas* sp. was identified (Chalmers 1992).

Trichomonosis in wild passerines has been infrequently reported until recent years. An outbreak affecting house finches, house sparrows and American goldfinches, contemporaneous with American mourning dove mortality (*Zenaida macroura*), occurred in Kentucky, Ohio and Indiana in the autumn of 2002. A combination of trichomoniasis and West Nile virus infection was diagnosed as the cause or mortality (estimated total of 200 birds) although the relative importance of these agents was not described (NWHC 2002). Gerhold et al. (2008) examined a *T. gallinae* isolate from a house finch in Kentucky in their molecular characterisation study of multiple strains of the parasite from a variety of avian host species, and found that the isolate clustered with those from sympatric columbiform species. Anderson et al. (2009) described endemic trichomonosis affecting passerine and corvid species submitted to a wildlife hospital in northern California. Their study found a high case-mortality rate and no evidence for a carrier state in the house finch, as can occur in columbiform species, but an overall low prevalence of infection of 1.7% (59/ 2971 birds). Forzán et al. (2009; 2010) recently reported trichomonosis as an EID of wild finches in eastern Canada, affecting wild purple finches and American goldfinches, describing 8 disease incidents that occurred during 2007 and 2008.

Widespread mortality due to trichomonosis in free-ranging finch species in Great Britain had not been reported in the literature prior to 2005. During the hot summer of 1976, chaffinch and greenfinch deaths occurred at a site in close proximity to a racing pigeon loft with a history of trichomonosis. Clinical signs included difficulty in swallowing: therefore trichomonosis of the upper alimentary tract was suspected although no pathological examinations were performed to determine the aetiology (D. Glue, *pers. comm.*).

In February 1993, 3 greenfinches found dead at a site in Oxfordshire were examined post mortem at the Institute of Zoology (IoZ). Morbidity affecting multiple collared doves (*Streptopelia decaoacto*) with signs of difficulty in swallowing and neck swelling had been recently reported at the same site. Necrotic ingluvitis was observed in all cases with no other significant gross lesions detected. Microbiological examination of oesophageal lesions found no evidence of salmonellosis but flagellate organisms with morphology characteristic of trichomonads were observed on a wet preparation from lesions in a single case. Trichomonosis was considered to be the cause of death for the finches examined. No columbiform birds were examined from the site although trichomonosis was suspected (Kirkwood JK and Macgregor S, *unpublished data*).

These reports suggest that localized spill-over of trichomonad parasites from columbid hosts to finch species has occasionally occurred in Great Britain in the past and that factors including close species proximity and climate may have influenced this transfer. However, wild bird disease surveillance by a variety of diagnostic centres found no evidence for continued transmission of the parasite between British finches leading to widespread disease after either of these reports until the emergence of trichomonosis as a cause of significant finch mortality in 2005, progressing to epidemic mortality in 2006 and 2007: the epidemiology and impact of this emerging infectious disease of British Passeriformes is described in this thesis.

Species identification and molecular epidemiology of *Trichomonas gallinae*

Strain variation in *T. gallinae* is well known and infection may be asymptomatic, subclinical or result in clinical disease (Cole 1999a). Host immunity and factors such as signalment also influence the outcome of infection (Cole 1999a). Birds suffering from trichomonosis typically develop necrotic ingluvitis (Forrester et al., 2008; Narcisi et al., 1991) although some *T. gallinae* strains are known to have tropisms for other organs, for example the Jones-Barr strain frequently results in hepatic lesions in columbiform species (Perez-Mesa et al., 1961).

Historically, morphological features have been used to help differentiate between the Trichomonadidae (Benchimol 2004), in combination with a consideration of the host species and the anatomical site from which the protozoa was isolated. For example, trichomonad parasites recovered from the upper and lower alimentary tract of birds have been generally considered to be *T. gallinae* and *Tetratrichomonas gallinarum* respectively (Gaspar da Silva et al., 2007).

In recent years, molecular epidemiological techniques have been employed to determine the phylogenetic relationships between members of the Trichomonadidae. Isoenzyme electrophoresis and restriction enzyme analyses have been performed to distinguish between strains of *T. gallinae* from different avian species, and strains of variable virulence (Nadler et al., 1988; Mattos et al., 1997; Knispel 2005).

Comparative sequence analysis has shown the internal transcribed spacer (ITS) 1/ 5.8S rRNA/ ITS 2 region to be useful for phylogenetic analysis to identify interspecific variation within the Trichomonadidae family (Felleisen 1997; Walker 2003; Kleina et al., 2004). Since the ITS regions are non-coding, greater levels of genetic heterogeneity are predicted in their sequence data as compared with coding genes.

Kleina et al. (2004) analysed sequence data of the ITS1/ 5.8S rRNA/ ITS 2 region from 36 strains collected from 14 species belonging to the Trichomonadidae and Monocercomonadidae which included *Trichomonas gallinae* and *Tetratrichomonas gallinarum* parasites derived from avian hosts. In their study, *T. gallinae* clustered in

Clade III, with closest identity to strains of *T. tenax* and *T. vaginalis* from humans, whilst the *T. gallinarum* isolates (from a chicken (*Gallus gallus*) and duck (*Anas platyrhynchus*)) were placed in Clade I.

Cepicka et al. (2005) sequenced the ITS1/ 5.8S rRNA/ ITS 2 region of multiple *Tetratrichomonas gallinarum* isolates and found evidence for significant intraspecific diversity between strains and cryptic species complexes.

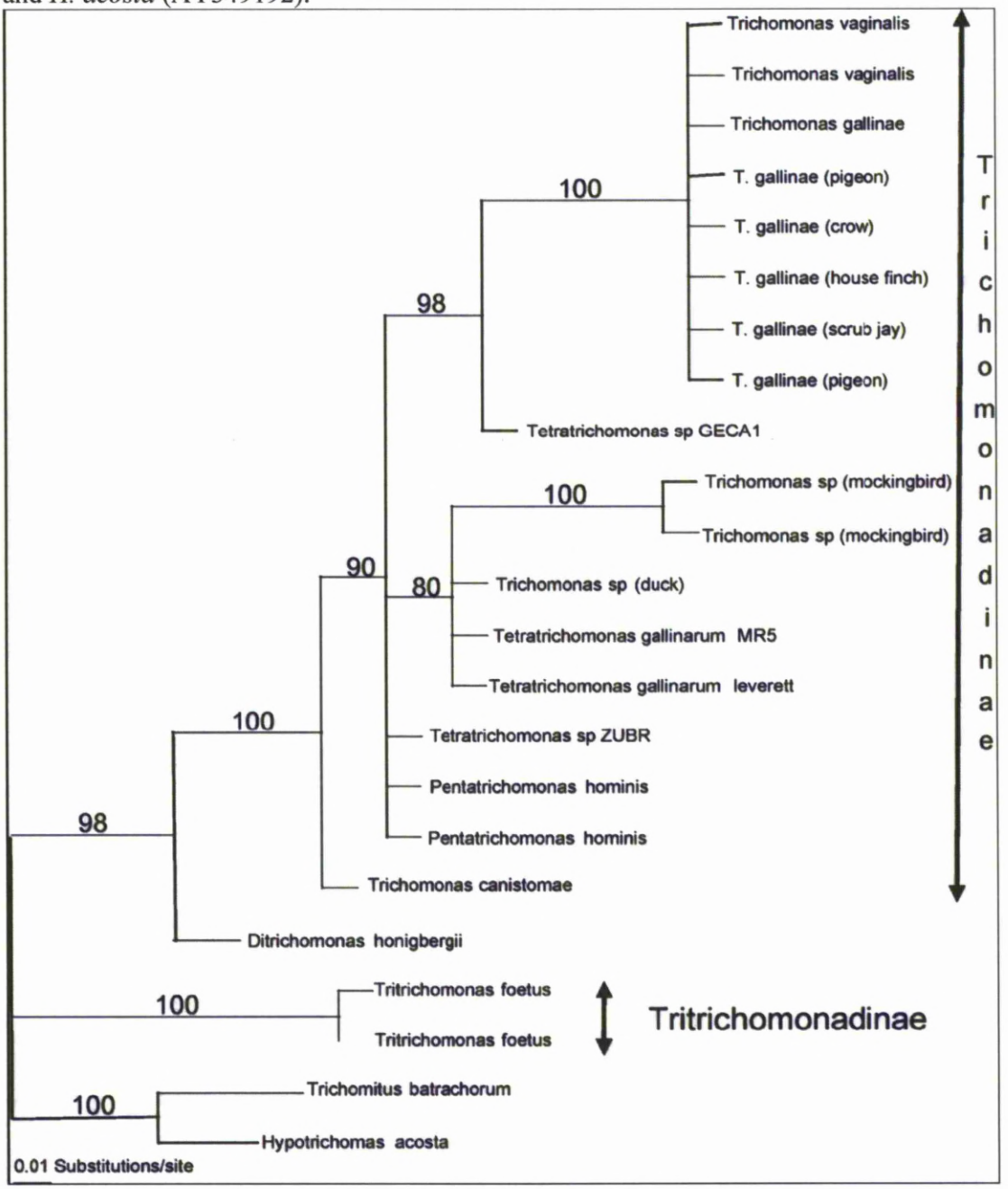
Gaspar da Silva et al. (2007) used this technique to evaluate genotypic variation between 24 isolates of *T. gallinae* from Mauritian columbid species and concluded that the ITS1/ 5.8S rRNA/ ITS 2 region is a useful species marker for the parasite. Sequence data from all isolates of Mauritian pink pigeons (*Columba mayeri*) and Madagascar turtle-doves (*Streptopelia picturata*) were found to be identical to one another and that of the strain (AY349182) from a pigeon host described by Kleina et al. (2004), and differed by 3 base pairs from the Rivolta (1878) strain (U8B614) from a pigeon host. This study concluded that the lack of sequence variation in this region could relate to the relatively recent introduction of *T. gallinae* into the Mauritian island avian fauna. Since the Rivolta (1878) strain had been maintained in long term axenic culture sequence change may have occurred, therefore the authors highlighted the need for further studies of *T. gallinae* parasites from a wider geographical region (Gaspar da Silva et al., 2007).

When Gerhold et al. (2008) examined the ITS1/ 5.8S rRNA/ ITS 2 region of 42 *T. gallinae* isolates collected from a wider geographical region from 9 North American States, these authors found evidence for 2 different species within a *T. gallinae* morphologic complex and multiple nucleotide polymorphisms between isolates. The first group, which contained all rock pigeon-derived isolates in the study, had high sequence identity to the original *T. gallinae* strains available in Genbank from rock pigeons in the U.S.A. (AY349182 Kleina et al., 2004; U86614 Felleisen 1997) and the Mauritian columbids (EF208019 Gaspar da Silva et al., 2007), whilst the second *Trichomonas vaginalis*-like group, isolated from white-winged doves (*Zenaida asiatica*) appeared to be a unique trichomonad species.

Anderson et al. (2009) analysed sequence data from the ITS1/ 5.8S rRNA/ ITS 2 region of *T. gallinae* isolates cultured from passerine species in northern California. Isolates from the house finch and corvids (Scrub jay (*Aphelocoma californica*) and American crow (*Corvus brachyrhynchus*)) were identical to the *T. gallinae* strain (AY349182 Kleina et al., 2004) from a rock dove and therefore cluster with Clade III described by these authors'. However, the *T. gallinae* isolate obtained from a mockingbird (*Mimus polyglotttos*) was quite divergent and clustered with *T. gallinarum* in Clade 1 described by Kleina et al. (2004) (Figure 1.1).

Comparative sequence analyses have also employed coding DNA genes to examine interspecific and intraspecific variation within the Trichomonadidae, including the small subunit (SSU) rRNA (Gunderson et al., 1995; Edgcomb et al., 1998; Delgado-Viscogliosi et al., 2000; Cepicka et al., 2005), iron containing superoxide dismutase gene (Viscogliosi et al., 1996), glyceraldehyde-3-phosphate dehydrogenase gene (Viscogliosi et al., 1998; Gerbod et al., 2004) and the alpha-tubulin gene (Gerhold et al., 2008). Gerhold et al. (2008) performed phylogenetic analysis of sequence data from the coding SSU rRNA and alpha-tubulin gene in *T. gallinae* strains and determined that the results support the 2 groupings of *T. gallinae* and *T. vaginalis*-like strains as per the non-coding ITS1/ 5.8S rRNA/ ITS 2 region.

Figure 1.1 reproduced from Anderson et al., (2009). Phylogenetic analysis of 100 5.8s rRNA nucleotides. Maximum Likelihood tree with Bayesian bootstrap values. Names in ( ) indicate sample origin. Additional Genbank samples used include: *Trichomonas vaginalis* (AY34985, AY34986), *T. gallinae* (U86614), *Tetratrichomonas* sp. (AY886826), *Trichomonas* sp. duck (AF236105), *T. canistomae* (AF784786), *Tetratrichomonas gallinarum* leverett (AY349180), MR5 (AY349181) and ZUBR (AY886841), *Pentatrichomonas hominis* (AY245137, U86616), *D. honigbergii* (AY349188), *Trichomonas foetus* (AY48567, AY485679), *T. batrachorum* (AY349193) and *H. acosta* (AY349192).



Random amplified polymorphic DNA (RAPD) analyses utilise multiple non-specific primers (c. 10 nucleotides) to identify variation between isolates (Soll 2000) that occur due to point nucleotide substitutions which are often selectively neutral and so do not necessarily imply phenotypic variation. RAPD analyses are prone to inconsistent results therefore reproducibility of findings must be confirmed through multiple test replicates and methodologies must be standardized (Soll 2000). RAPD analyses have been used to study interspecific (Felleisen 1997) and intraspecific variation between trichomonad parasites (Vanácová et al., 1997). Gaspar da Silva et al. (2007) were the first authors to use RAPD analyses to study genotypic heterogeneity within *T. gallinae* strains and found that variation exists according to avian host species and island region. Sansano et al. (2008) used RAPD analysis on *T. gallinae* isolates collected from 5 Spanish raptor species and found genotypic variation, although no consistent relationship with the avian host species was found.

In this thesis, both morphological and molecular techniques are used to confirm the parasite species identity responsible for finch trichomonosis. A combination of phylogenetic sequence analysis, from coding genes and non-coding regions, and RAPD analyses are used to investigate the likely origin of the trichomonad parasite in finch populations and to examine evidence for strain variation within British bird populations. Published techniques are modified and novel approaches developed to identify an improved and robust method for differentiation of *T. gallinae* strains.

VIRAL

i. Avian poxvirus

Avian pox infection is a well known disease of captive and wild birds caused by dsDNA viruses (150-250 nm x 265-350 nm oval or brick-shaped virions) in the genus *Avipoxvirus*, family Poxviridae (McFerran et al., 1993; van Riper et al., 2007). Avian poxviruses are known to have variable host specificity with strain classification based on the birds typically affected (e.g. fowl, canary, pigeon and falcon poxvirus) (Karstad 1971; Francki et al., 1991; Ritchie 1995); some strains affect a range of species whilst others appear to be species-specific (Kirmse 1967; Hansen 1999).

Avian pox typically causes discrete, proliferative and 'wart-like' lesions on the featherless regions of the head (particularly around the eyes and commissures of the beak), the legs and feet. This clinical presentation is frequently self-limiting, with lesions restricted to the skin, and described as 'dry' infection. The incubation period and duration of avian poxvirus infection is variable but affected birds with mild lesions frequently recover and this is considered to be the most common situation in wild birds (Simpson et al., 1975). However, severe avian pox infection may compromise vision, the ability to feed, or lead to secondary bacterial or fungal infection, leaving wild birds vulnerable to predation (Hansen 1999). 'Wet' avian pox infection refers to cases with diptheritic lesions in the alimentary or respiratory systems, in addition to the integument, and has been infrequently reported in wild birds although this may because the internal disease is more cryptic than the simply observed cutaneous presentation (Hansen 1999). Systemic septicaemic avian pox infection has been described in captive finches and canaries (Ritchie 1995). Differential individual susceptibility to infection affecting the severity of the disease is thought to be dependent on factors such as age, with juveniles most susceptible, immunocompetence, species, season and local environment (Ritchie 1995).

Avian poxviruses are mechanically transmitted through direct or indirect contact, through biting insect vectors, or less commonly via aerosol infection (Ritchie 1995). Mosquito species are considered to be the most common vectors of infection, in addition to diptera, midges and mites (McFerran et al., 1993). Climatic factors, such as temperature and relative humidity, influence mosquito population cycles and in temperate climates these vector populations typically peak in late summer, coincident with an increasing population of first-year birds näive to the infection; these factors chiefly dictate the seasonality of avian pox infection observed in wild birds which tend to peak in the summer and early autumn (van Riper et al., 2007). Climatic factors such as temperature and relative humidity also influence environmental survival of the virus (Hansen 1999). Viral transmission through direct contact between birds, for example congregation at bird feeders, might occur during cooler months (Hansen 1999).

Avian poxvirus transmission is density-dependent and conditions which favour close and sustained contact between birds typically favour spread of infection. In captive birds, avian pox has been most commonly observed in game bird rearing, captive breeding programmes and quarantine facilities. High morbidity and mortality has occurred, for example the northern bobwhite (*Colinus virginianus*) in the United States (estimated morbidity of 2% and 0.6-1.2% mortality) (Davidson et al., 1980) and red-legged partridges (*Alectoris rufa*) in Spain (estimated morbidity of 40% of juveniles and 2.9% of adults) (Buenestado et al., 2004).

In wild birds, severe infection has been reported in wildlife rehabilitation centres where concurrent immunosuppression, mixing of species and aerosol transmission support outbreaks of disease (Wheeldon et al., 1985; Hansen 1999). An outbreak of avian pox infection was reported in peregrine falcons (*Falco peregrinus*) that were part of a reintroduction programme in Germany (Krone et al., 2004).

In free-ranging wild birds, avian pox most typically results in sporadic infection at low prevalence (0.5-1.0%) affecting individual birds where a stable co-evolutionary relationship exists between the host and virus (van Riper et al., 2007). Introductions of avian poxviruses to island populations have led to the greatest disease prevalence, sometimes with negative effects at the population level, affecting immunologically näive island endemic species, for example 35% prevalence of some bird species in Hawaii (Warner 1968; van Riper et al., 2002), 28% prevalence in the Galapagos islands (Vargas 1987) and >10% prevalence in New Zealand (Westerkov 1953). Avian pox has recently been diagnosed for the first time in the Canary Islands and concern has been expressed regarding this disease threat to island endemic avifauna (Medina et al., 2004; Smits et al., 2005).

Avian pox infection, including outbreaks of disease, has been reported in American mourning dove (*Zenaida macroura*) and house finches at garden bird feeding stations with high bird density in North America (Hansen 1999; McClure 1989).

Worldwide, avian pox infection in wild birds has been most frequently reported in Galliformes, Passeriformes and colonial-nesting marine birds (Phaethontiformes and Procellariiformes) although infection has been reported in 278 species from 70 families and 20 orders to date (Hansen 1999; van Riper et al., 2007).

In Great Britain, sporadic reports of poxvirus infection exist from multiple wild bird families and orders (van Riper et al., 2007) but local outbreaks of disease are rare. Since the 1950s, avian pox infection has been reported to affect numerous bird species in Great Britain that visit garden habitats including the blackbird (*Turdus merula*), carrion crow (*Corvus corone*), chaffinch, dunnock, greenfinch, goldfinch (*Carduelis carduelis*), house sparrow, jackdaw (*Corvus monedula*), starling and wood pigeon (Jennings 1954; Edwards 1955; Poulding 1960; Keymer et al., 1964, Blackmore et al., 1969; Pennycott 2003). Avian pox has also been reported across continental Europe in a number of other passerine species that are present in Great Britain (van Riper et al., 2007).

Internationally there are relatively few reports of avian pox infection in Paridae species (Gruber et al., 2007; van Riper et al., 2007). Avian pox infection has been previously described in the great tit in Germany and Norway (Polowinkin 1901, Holt et al., 1973). In Norway in the early 1970s, epizootic outbreaks of avian pox occurred for the first time in wild terrestrial birds affecting multiple species, although the great tit was most commonly affected. Incidents peaked in the autumn and winter months and case mortality rate was thought to be low. Lesions were restricted to the head and ranged from small nodules to large spherical tumour-like proliferations of <20mm diameter with a capsule and necrotic custard-yellow caseous material on transverse section (Holt et al., 1973). More recently, a single incident of avian pox infection occurred in Austria, October 2005, where 4 great tits in a flock of 15 birds were seen to be affected (Gruber et al., 2007). In 2007, a Hungarian survey of 1819 great tits by licensed ringers observed nodular and proliferative lesions on the head and eyelids of 15 birds that were confirmed as avian poxvirus infection (Palade et al., 2008).

There have been no reports of avian pox infection in Paridae species in Great Britain in the peer-reviewed literature or through disease surveillance since the mid-nineties in Great Britain (IoZ, Wildlife Veterinary Investigation Centre, Scottish Agricultural College, VLA, *unpublished data*).

TOXINS

i. Aflatoxin

Aflatoxins (AFs) are a group of compounds whose metabolites have been demonstrated to have carcinogenic, immunosuppressive, hepatotoxic and other pathological effects through impairment of protein synthesis and interaction with metabolic pathways (Pier et al., 1970; Pier 1992; Quist et al., 2000). AFs naturally fluoresce under long-wavelength ultraviolet (UV) light and this physical property is used to classify the 4 major toxins: Aflatoxin B_1 (AFB_1) and Aflatoxin B_2 (AFB_2), bifurano coumarins fused to cyclopentanone, fluoresce blue under UV; aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2), bifurano coumarins fused to lactone, fluoresce green (Gourama et al., 1995). AFB_1 is the most potent and commonly produced of the AFs; the toxicity of aflatoxin G_1, B_2 and G_2 relative to AFB_1 is approximately 50, 20 and 10%, respectively (Smith et al., 1991). The subscripts $_1$ and $_2$ refer to the chromatographic mobility pattern of the toxins on thin layer chromatography plates (Gourama et al., 1995).

The fungal species *Aspergillus flavus, A. parasiticus* and *A. nomius*, are responsible for the production of AFs (Pitt et al., 1997). These fungi are ubiquitous in the air, soil and other natural substrates. AFs are therefore considered natural contaminants of a variety of agricultural products, with corn, peanuts, cottonseed, and other grain crops being most frequently contaminated (Gourama et al., 1995; Creekmore 1999); many of these foodstuffs are components of supplementary food sold for wild bird consumption in Great Britain.

AFs were first identified when they were found to be a cause of acute toxicity in commercial turkeys in the 1960s, the condition being known as 'Turkey-X disease' (Blount 1961). Aflatoxicosis has been documented since in a variety of mammals, fish

and birds. Although birds appear to be the most susceptible, there is significant variation between avian species in their susceptibility to AFs (Creekmore 1999). Also, susceptibility to the effects of AFs varies with age, sex and nutritional status, with young birds likely to be the most significantly affected (Creekmore 1999; Maia et al., 2002). The acute effects of AF toxicity are characterised by hepatic injury, coagulopathy, haemorrhage, icterus and death (Pier 1992). Chronic, low level exposure to AFs has been shown to be associated with a range of more insidious effects, such as reduced weight gain, suppression of the immune system, interference with reproductive function and neoplasia (Pier 1992; Sharma 1993; Ortatatli et al., 2002; Verma et al., 2004).

Much research has been performed on the effect of AF exposure on domestic farmed bird species (Muller et al., 1970). Given the susceptibility of farmed turkeys to AFs, concern was raised over similar potential effects on wild turkeys (*Meleagris gallopova silvestris*) in the U.S.A.. Experimental AF exposure in this species led to reduced weight gain and feed consumption, impaired cell-mediated immunity and mild liver damage (Quist et al., 2000). Feeding trials were performed on wild game bird species to examine the degree of interspecific variation in their response to AF exposure. Dietary AF concentrations of 1250, 2500 and 5000 μg/kg (ppb) were fed for 3 weeks; these concentrations were chosen since they represent the range in chickens that have been found to result in no adverse effects, mild effects and severe effects respectively. In order of relative susceptibility to the effects of AF, ring neck pheasants (*Phasianus colchicus*) were most affected, followed by the domestic chicken and bobwhite quail; chukar partridge (*Alectoris chukar*) and the Japanese quail (*Coturnix coturnix japonica*) were relatively resistant. Significant mortality was experienced by ring neck pheasants and bobwhite quail at dietary AF levels exceeding 1250 μg/kg (Ruff et al., 1990; Huff et al., 1992; Ruff et al., 1992). It is noteworthy that the relative susceptibility of the 2 quail species differed markedly.

There is a paucity of data available on the susceptibility of passerine species to AF exposure. Experimental dietary exposure of wild-caught northern cardinals, Family Cardinalidae, to AFs in a 28-day experiment found that acute toxicity and mortality

occurred in <20% of birds that received 100 μg/kg AF in their diet and >47% of birds that received >100 μg/kg AF in their diet. Importantly, subclinical effects of AF exposure were noted at lower toxin concentrations, for example, white blood cell profileration was 'greatly suppressed' at 50 μg/kg AF, indicating that immune function compromise may occur with chronic low level exposure (Henke et al., 2004).

The relative susceptibilities of garden bird species in Great Britain to the toxic effects of AFs have not yet been determined, and it is clear from findings in other bird species, that they could not be predicted, even from those of apparently similar species. Dumonceaux et al. (1994) noted that stressed birds (species not specified) and those on a poor diet were more susceptible to the same toxin challenge than healthy, well-fed birds. Thus, garden birds may be most vulnerable to the effects of AF exposure during the winter months, when reduced natural food availability and inclement weather can occur. Birds may similarly be 'stressed' through the physiological demands of the breeding season, or during migration, when they rely on stored energy reserves.

In recent years, increasing focus has been placed on the study of aflatoxicosis in free-ranging wildlife species. Mass mortalities of several waterfowl species due to acute aflatoxicosis have been reported in Texas (1970s) and Louisiana (1998-1999), U.S.A.. Deaths occurred during the autumn/winter season and were attributed to the birds feeding on waste crops (e.g. peanuts, corn) in agricultural fields (Robinson et al., 1982; Cornish et al., 1999; Creekmore 1999). Robinson et al. (1982) reported 2 mortality incidents affecting primarily snow geese (*Anser caerulescens*) and mallards (*Anas platyrhynchos*) respectively. Clinical signs reported in the affected waterfowl were relatively non-specific and include apparent blindness, unresponsive behaviour, depression, weakness, inability to fly and resting in a sitting position. Gross examination revealed hepatic pallor; histopathological examination confirmed acute hepatic necrosis and biliary proliferation. Analysis of a pooled sample of oesophageal and proventricular contents from affected birds in the first incident yielded 500 μg/kg AFB_1 (dry weight) whilst the same sample types from individual birds from the second incident yielded levels from 10 to 250 μg/kg AFB_1 (dry weight). Robinson et al. (1982) found 110 μg/kg

AFB_1 in a sample of peanuts taken randomly from the field of the second incident. Cornish et al. (1999) analysed samples of corn from fields where a variety of goose species were affected by acute aflatoxicosis and found levels as high as 8200 μg/kg AF. Recently, concern has been expressed for the significance of chronic low level exposure of AFs to wild bird species either through supplementary, agricultural or wild seed sources (Schweitzer et al., 2001).

There are no reports in the literature of acute mortality due to aflatoxicosis, or other mycotoxicosis, in free-ranging wild birds of any species in Great Britain. Although no evidence, either anecdotal or scientific, is available on AF exposure of birds in Great Britain, such exposure has been repeatedly raised as a possible cause of morbidity or mortality of garden birds provisioned with commercially-available food by householders. It is possible that chronic exposure to low level AF contamination may impair immune function and predispose garden birds to infectious disease. However, since overt clinical signs of aflatoxicosis would not be predicted in these cases, research is required to determine whether garden birds are exposed to AFs in Great Britain, and to appraise any pathological significance that this may pose.

EU Directive 1999/29/EC (Anon 1999b) stipulates the legal maximum permissible level (MPL) of AFB_1 of 20 μg/kg for groundnuts (peanuts), maize and maize products used for animal feeding stuffs, including those for wild animals, and this is implemented in UK legislation (Anon 2004a; 2004b; 2004c; 2004d). The MPL for sunflower seeds used for animal feeding stuffs is AFB_1 50 μg/kg. There is currently no MPL for AFB_2, AFG_1 and AFG_2.

In 2002, the Advisory Committee on Animal Feedingstuffs (ACAF) of the Food Standards Agency (FSA 2003), considered whether to adopt a specific MPL for AFs in wild bird foods. Human food safety concerns were a major driver in this review since there is an order of magnitude disparity between the MPL for nuts used for human consumption (AFB_1 2 μg/kg) and wild birds. Food products that are not clearly labelled for wild bird consumption may be inadvertently consumed by humans with concomitant

health concerns. ACAF identified a research need and concluded that "*there has been no scientific risk assessment or review to determine if* [current MPL] *are appropriate to wild birds*" and consulted interested parties on the issue in 2002. The consultation (FSA 2003) prompted contributions from garden bird food industry representatives and ornithological non-governmental organisations, amongst others. Several options were considered for action, for example, to bring the MPL for wild bird foods in line with those currently applicable for poultry chicks (AFB_1 10 μg/kg) or human (AFB_1 2 μg/kg) consumption. Alteration of the AFB_1 MPL would have economic and human health implications, which may differ in developed and less-developed countries, particularly China and Argentina, which require risk assessment (Felicia 2004). In 2006, The Standing Committee on the Food Chain and Animal Health, Animal Nutrition Section, Brussels, considered the information from the UK consultation (EC 2006). The European Committee concluded that there was a need for further discussion before introduction of a dedicated MPL for wild bird food products and suggested that a voluntary labelling initiative across the garden bird food industry be considered.

Since 1983, members of the BirdCare Standards Association (BSA) in the UK have followed the precautionary principle and follow a code of standards whereby only peanuts that have been screened and found to contain nil detectable levels of AF (BSA 2009) are sold. Other non-BSA member companies have also adopted the same policy. However, much food purchased for garden bird provisioning in Great Britain is bought from retailers outside these associations which are governed by the statutory MPL and which frequently have no clear labelling on the quality control procedures for mycotoxin testing that have been employed.

ii. Ochratoxin A

Ochratoxin A (OA) is one of a group of fungal metabolites that are classed as dihydroisocoumarins (Smith et al., 1991). OA is the most toxic of the ochratoxins and is most frequently produced by *Aspergillus ochraceus* (van der Merwe et al., 1965), although other species of *Aspergillus* and several species of *Penicillium* also produce the toxin (Smith et al., 1991; Santin 2005).

OA, as with AFs, can lead to acute toxicity or insidious multi-system effects following chronic, low dose exposure. OA is principally nephrotoxic; kidneys of chickens affected by ochratoxicosis are enlarged, appear much paler than normal (Huff et al., 1974; Dwivedi et al., 1984a) and uric acid precipitates may be grossly visible (Chang et al., 1981). Distension and hypertrophy of the proximal convoluted tubules, combined with thickening of the glomerular basement membrane, may be seen on histopathological examination of affected birds (Dwivedi et al., 1984a).

As with AFs, the toxic effects of OA vary, depending on the dose ingested, the age, species and individual involved (Huff et al., 1974; Chang et al., 1981). The majority of research on ochratoxicosis has focused on poultry and game bird species.

Clinical signs of ochratoxicosis observed in poultry species (chickens and turkey poults) under experimental exposure conditions include a progression of symptoms from listlessness, huddling and diarrhoea, followed by ataxia, neurological symptoms and fine muscle tremors, to prostration and finally death (Huff et al., 1974; Chang et al., 1981).

Experimental exposure of 4 game bird species to diets containing 1, 2 or 4 mg/kg (ppm) OA for 3 weeks found evidence for interspecific variation in the susceptibility to the toxin; as with AFs, these authors' selected this range of dietary OA concentrations since they have been shown to result in nil detectable to severe side effects in chickens. On the 4 mg/kg OA diet, 10% mortality occurred in chukar partridge and 5% in the ring-neck pheasant; no mortality resulted in the bobwhite or Japanese quail (Ruff et al., 1990; Huff et al., 1992; Ruff et al., 1992). Reduced weight gain occurred in the chukar partridge on the 4 mg/kg OA feed (Ruff et al., 1990) and in turkey poults fed the 4 and 8 mg/kg OA feed (Chang et al., 1981)

Chang et al. (1981) observed a lymphocytopaenia in turkey poults fed 4000 and 8000 mg/kg OA. Dwivedi et al. (1984a,b) also found that OA caused "*a drastic reduction in the lymphoid cell population [including immunoglobulin-containing cells] in the*

immunological organs" (bursa of Fabricius, thymus, spleen, caecal tonsils and Peyer's patches) in broiler chicks and a reduction in total serum immunoglobulin levels, and Huff et al. (1974) observed a regression of the bursa of Fabricius in chickens fed OA.

Experimental challenge of chicks with *E. coli* alone resulted in 14.3% mortality; in contrast bacterial challenge combined with a diet containing 2 mg/kg OA resulted in 35.7% mortality (Kumar et al., 2003).

In common with AF, OA has effects on reproduction, such as reduced egg production, poor eggshell quality, decreased embryo viability and poor hatchability (Devegowda et al., 2005).

No experimental studies have been performed to evaluate the susceptibility of passerine species to OA and there have been no confirmed cases of ochratoxicosis in wild birds, in Great Britain or overseas.

Co-contamination of feedstuffs with AF and OA can occur (Wyatt 2005). Experimental studies indicate a synergistic effect of combined AF and OA exposure, for example growth impairment in broilers was significantly greater than the expected additive effect of the 2 toxins (Huff et al., 1981; Verma et al., 2004).

There is currently no MPL for OA in animal feedingstuffs; an EU Commission Recommendation 2006/576/EC (Anon 2006) provides guidance values for a maximum level of 100 μg/kg OA in poultry feedstuffs. Minimal attention has been paid to the significance of OA exposure of wild birds in Great Britain and no voluntary accreditation schemes specifying permissible OA levels have been adopted by the industry.

SURVEYS OF MYCOTOXIN IN WILD BIRD FOODS

In the U.S.A., the possibility that grain products condemned as unsuitable for domestic animal consumption, due to their AF residue concentration, may be used for

supplementary feeding of wildlife has been raised (Fischer et al., 1995; Quist et al., 2000). Measures to mitigate mycotoxin production, both pre- and post-harvest, include integrated pest control and bioengineered crops (Felicia 2004); mycotoxin binders (e.g. montmorillonite, clinoptilolite) are commercially available for use in pelleted poultry feedstuffs (Oguz et al., 2000; Shi et al., 2006) but are unlikely to be of use with whole nut or grain mixes used for garden bird food.

Several studies have been performed to determine AF concentrations in feed commercially available for wildlife. In Georgia, U.S.A., corn samples sold as wildlife feed from a variety of retail sources were tested for AF and 10% (3/31 samples) had detectable AFs, the highest level being 380 μg/kg total AF (Schweitzer et al., 2001). In Brazil, a survey of pet feeds, including 30 bird feeds, found 12% (12/100 samples) had detectable AFs, 5 of which exceeded the national MPL of 50 μg/kg. Peanuts were used in the bird foods and all samples containing peanuts were positive for AFs (Maia et al., 2002). Wild bird seed mixes sold through a variety of retail outlets (grocery stores, pet shops, grain co-operatives) from locations throughout Texas, U.S.A., were also tested. Seventeen percent of the 142 samples contained AF concentrations exceeding 100 μg/kg, with the range of values from 0 to 2780 μg/kg (Henke et al., 2001). A study in Slough, England, screened 100 pet food samples including 15 samples of wild bird food of various types and found 1 positive for AFs (Scudamore et al., 1997). This was an open 225g plastic string bag of peanuts, from which 3 samples were taken that yielded values of 160, 360 and 370 μg/kg AFB_1, compared with another bag of peanuts from the same store with levels around 4 μg/kg AFB_1. This work illustrates the difficulties in screening for AFs due to heterogeneity in toxin production and the need for adequate sampling protocols (Scudamore et al., 1997).

Fewer studies have been performed to determine the levels of OA in wild bird food. Oberheu et al. (2001c) tested samples of a 50/50 mix of maize and sorghum from supplemental feeders provided for game birds in Texas and Oklahoma (U.S.A.) and found OA concentrations ranged from 5 to 109.9 μg/kg; the authors noted that, whilst feeders may be a source of OA for wild birds, these levels were not close to the range

shown to exert negative effects in experimental trials. In the British study carried out by Scudamore et al. (1997), a low level (6 μg/kg) of OA was found in 1 of the 15 samples of wild bird food tested.

AFLATOXIN AND OCHRATOXIN A PRODUCTION

The fungi that produce mycotoxins can invade grain and other crops, such as peanuts, in the field and/or during storage, transportation and processing, and can produce toxins at any stage, under certain conditions (Santin 2005).

It is important to note that not all strains of *Aspergillus* spp. produce AFs (Smith et al., 1991), so the presence of mould in a crop does not necessarily indicate the presence of AFs. Similarly, fungal growth is not always visible to the naked eye (Santin 2005), and thus AFs may be present in crops without an obvious presence of mould.

Aflatoxin production varies dependent on a number of physical factors, including ambient temperature, relative humidity, pH, substrate etc. (Gourama et al., 1995). Whilst conditions for *Aspergillus* spp. growth are optimal between 36-38°C, growth can occur between 6-46°C. Similarly, the optimal temperature range for AF production is thought to be between 25-28°C, although production can occur over a wider range of values. Relative humidity values of 77-90% are required for AF production, although *Aspergillus* spp. growth can occur at a lower R.H. if the moisture content ranges between 12-17% (Salunkhe et al., 1987; Gourama et al., 1995; Oberheu et al., 2001b). Consequently, although *Aspergillus* spp. are widely distributed, problems with AF production most frequently occur in regions with hot and humid climates.

However, the results of a recent study on the effect of environmental conditions and type of storage container on AF production demonstrated the potential for AF production in stored cereals under a variety of climate conditions (Thompson et al., 2000). Small samples of corn were collected and screened to confirm that they were negative for AFs (detectable threshold 1.0 μg/kg) at the onset of the trial. Samples were then stored in a constant climate for a period of 90 days at either 29-32°C or 14-18°C, and relative

humidity value of 85-88% or 35-40%. Whilst only 8% of these samples contained AF levels >50 μg/kg at the end of the period, AF was produced under all storage conditions and climatic condition combinations (Thompson et al., 2000). This study raises the possibility that AF production can occur in a temperate country such as Great Britain.

Oberheu et al. (2001b) also conducted a study in the field to determine the relationship between weather conditions and AF production in agricultural grain (sorghum and corn) in supplemental feeders provided for northern bobwhite quail, in Texas and Oklahoma, during winter months. Mean monthly values of total AF varied between 0.57-15.47 μg/kg and the highest concentration of AF detected in an individual sample was 157 μg/kg. A significant increase between the pre-storage and first month levels of AF was identified, consequently, the authors' concluded that their study gave evidence that AF can be produced in supplemental feeders in the U.S.A. (Oberheu et al., 2001a). No similar research has been performed to date under climatic conditions in Great Britain.

Ochratoxins are known to be commonly produced under conditions of food product storage (Devegowda et al., 2005) and OA has been isolated from many agricultural commodities and products including cereals (maize, wheat, barley) and peanuts: common ingredients of wild bird food (Shotwell 1991; Yoshizawa 1991; Galvano et al., 2005).

OA is produced as a metabolite of *Aspergillus* spp. and *Penicillium* spp., the latter most adapted to temperate climates (Smith et al., 1991; Santin 2005), such as that in Great Britain. For example, *P. verrucosum* grows at temperatures between 0 and 31°C, inclusive, and OA production can occur over the whole range, with optimum production at 20°C (ICMSF 1996), whilst *A. ochraceus* grows at temperatures from 8 to 37°C and produces OA at 12 to 37°C, with optimum production at 31°C (ICMSF 1996).

Oberheu et al. (2001c), in their U.S.A. study of OA concentration in game bird feeders noted a positive correlation between OA concentrations and monthly mean relative

humidity which they interpreted as evidence to suggest that OA production did occur while the grain was in the feeders, although their sample size was too small to permit a quantitative estimate of OA production. As with AFs, no research has been performed to evaluate evidence for OA production in supplementary feed for wildlife.

Thompson et al. (2000) noted that 74% of the corn samples in their study became contaminated with AF in the third month of storage; this led the authors to suggest that the risk of AF consumption to wild birds could be reduced by limiting storage periods to a maximum of 2 months. The same authors noted that wildlife feeder designs that permit contact of food with moisture may provide conditions that favour fungal growth, particularly if feed is not replaced on a regular basis (Thompson et al., 2000). In the UK, Scudamore et al. (1997) found that poor storage of pet foods in conditions of high moisture content increased mould growth, however, no mycotoxin production (AF or OA) was found in the samples that were tested. Nevertheless, this study recommended that cereal-based foods should be stored in clean and dry conditions to prevent mycotoxin production (Scudamore et al., 1997).

In the absence of further data, the GBH*i* recommends sensible precautions regarding wild bird food storage: providing fresh food on a regular basis, purchasing food regularly and avoiding long-term storage prior to use, regularly cleaning feeders and removing stale food waste (UFAW 2005). Whilst this advice is provided, little is known of the feeding practices routinely used in Great Britain and research is required to determine how these might influence the likelihood of mycotoxin exposure to garden birds.

WILD BIRD POPULATIONS IN GARDEN HABITATS AND THE SIGNIFICANCE OF DISEASE

The index of wild bird populations is one of the UK government's key national indicators for its Sustainable Development Strategy which considers population trends for many native species. Most recent government assessments state that wild bird population indices have reduced since monitoring began in 1970 with an overall drop of

c. 51% and c. 20% for the farmland and woodland bird groups by 2007 (Defra 2009). In some cases, the cause of these species' declines are known (at least to a degree), such as altered land use and farming practices, but in many cases the causes of these declines is uncertain. Many of the bird species which frequent garden habitats, some of which frequently consume supplementary food at feeding stations, are included in both the woodland (e.g. specialist: blackcap *Sylvia atricapilla*, coal tit, generalist: blackbird, blue tit, bullfinch, chaffinch, dunnock, great tit, long-tailed tit, robin *Erithacus rubecula*, song thrush *Turdus philomelos*, wren *Troglodytes troglodytes*) and farmland (specialist: goldfinch, yellowhammer *Emberiza citrinella*, generalist: greenfinch, wood pigeon) categories (BTO 2006). Consequently diseases which adversely impact wild bird populations may influence the governmental indicators used as a barometer as sustainable development: an understanding of the relative significance of disease is essential for informed appraisal of these indicators.

Population declines in native song birds that traditionally visit garden habitats have been well-documented over recent decades. The song thrush and bullfinch have dedicated UK Biodiversity Action Plans and the current sharp decline in house sparrow and starling populations, species historically considered to be numerous, are reflected in their revision to red-listing status (JNCC 2008). Conservation-based ornithological organisations have invested considerable resources in trying to understand the causes of these declines and increasing attention has been paid to the significance of disease in this regard. For instance, a government-funded British Trust for Ornithology (BTO) report recommended the need for investigation of disease as a potential contributory factor to the house sparrow decline (Crick et al., 2002).

WILDLIFE GARDENING AND GARDEN BIRD FEEDING

Industrial and agricultural development has resulted in loss and degradation of available wildlife habitats across Great Britain for native bird populations (Vickery et al., 2004: Goddard et al., 2009). Consequently the importance of private gardens as habitats for wildlife species, and the need to augment their benefit for local biodiversity, is increasingly recognised (Cooper et al., 2007; Goddard et al., 2009).

For UK cities, a recent audit concluded that private gardens account for a significant proportion of available green space within urban city habitats, ranging from 21.8% to 26.8% of available space (Loram et al., 2007). Indeed, private garden habitats are estimated to cover an area of c. 500,000 hectares across England and Wales (Cannon et al., 2005); a significant land area when compared with the current 200 Royal Society for the Protection of Birds (RSPB) reserves across the UK of 130,000 hectares (RSPB 2010) and nature reserves in England of 120,000 hectares (Natural England 2010).

Wildlife gardening has been defined as "*any actions conducted in private or domestic garden to increase their suitability for wildlife, and this includes the provision of a diversity of resources*" (Davies et al., 2009). Wildlife gardening offers an opportunity with benefits to human well-being and native species conservation alike.

Concern has been raised that the public living within urban regions are physically separated from wildlife species which has resulted in disconnection, and even 'extinction', of their experience of nature (Miller 2005; Davies et al., 2009). Contact with the natural environment has been shown to have a range of positive health (e.g. promote longevity, stress-relief) and social benefits (e.g. reduced crime) (Davies et al., 2009). The British have a long held passion for bird-watching, encompassing dedicated enthusiasts and the general public alike. Contact with garden birds is frequently the most common interface between people and wildlife, particularly in urban and suburban areas. Feeding garden birds is a low cost recreational pursuit, perhaps unique in its accessibility from the home to people from all demographic groups, including the elderly and others with restricted mobility. The social importance of contact between wild birds and people is illustrated in the government document, A better quality of life: a strategy for sustainable development in the UK, 1999 (Anon 1999a).

Studies have demonstrated that significant proportions of some native bird species utilise garden habitats in the UK (Gregory et al., 1998; Mason 2000; Bland et al., 2004). The general public has adopted an approach of stewardship for the wildlife species that use

their garden habitats (Sainsbury et al., 2001). Public education and engagement with native wildlife fosters greater concern for biodiversity and conservation.

Government and non-governmental organisation initiatives promote 'wildlife-friendly' gardening practices and have increased available advice in recent years which has resulted in increased social and financial focus on the activity. For example, the government-funded London Biodiversity Partnership has formulated an Action Plan for Private Gardens which aims to "*to improve private gardens as habitat for a range of local wildlife*" (Biodiversity Action Reporting System 2008). The RSPB recently launched its Homes for Wildlife Scheme (RSPB 2008) with over 300,000 participants in 2008. Provision of seed (sunflower or millet based diets) for garden birds was the first in the RSPB's top ten list for activities in the first year of this scheme.

A recent comprehensive survey estimated that 98% of households across the UK have access to a garden of which 48% (c. 12.6 million households) feed wild birds. Overall 23% of houses with gardens use bird feeders for provision of supplementary food (c. 7.4 million households) (Davies et al., 2009). Moss (2000) similarly found that half of households questioned in a Royal Horticultural Survey provided food for garden birds. In the Feeding Garden Birds Survey (RSPB 2004) of c. 1000 respondents, over half had fed the birds within the past year and over one third regularly bought bird food. Gaston et al. (2007) in their questionnaire survey of residents in 5 UK cities determined that significant numbers of households partake in wildlife gardening and that providing food for birds was the most common action.

Based on summary figures for the national bird population, it is estimated that there is a bird feeder available for every 9 birds of species likely to use them in the UK (Davies et al., 2009). Kirkwood (1997) extrapolated from the data of Moss et al. (1998) who published that 15,000 tons of peanuts are used per annum in the UK; he determined that this sole food supply would be sufficient to fulfill the energy requirement of the entire breeding population of British greenfinches (Kirkwood et al., 1997). DeGraaf et al. (1975) estimated that households across the United States spent US$ 170 million each

year on birdseed as far back as 1974. The scale and complexity of garden bird feeding in the UK has increased markedly over recent decades with improved food quality and diversity (Chamberlain et al., 2005). These findings clearly illustrate the extensive scale of garden bird feeding which might plausibly influence the ecology of wild bird species across Great Britain. Indeed, feeding birds in garden habitats has been shown to influence the abundance of urban bird populations in a UK city (Fuller et al., 2008). Chamberlain et al. (2005) evaluated Winter Garden Bird Feeding Survey data from the UK and found a significant increase in the number of feeding stations available (considered a surrogate for the volume of food supplied) in garden habitats since the 1970s; their model predicted that this contributed to the observed increase in bird occurrence within private gardens for multiple species.

Feeding garden birds is thought to have numerous potential benefits for wild bird populations including increased overwinter survival (Orell 1989), improved nutritional condition (Grubb et al., 1990) and enhanced breeding productivity (Arcese et al., 1988). The potential for adverse effects associated with feeding garden birds exists. Concern has been expressed that birds may become reliant on food resources. Black-capped chickadees in North America were found to forage on natural food rather than become reliant on sunflower seeds when available (Brittingham et al., 1992a,b). The abundance of siskins and coal tits in a Scottish garden varied with Sitka spruce (*Picea sitchensis*) cone abundance; these birds were shown to use supplementary food in gardens only in years with poor mast crops (McKenzie et al., 2007). Supplementary food may be of poor nutritional quality, risk of predation may be increased and anthropogenic disturbance may be high in the vicinity of garden feeding stations (Davies et al., 2009). Congregation of garden birds at feeding stations, with increase intra- and interspecific rates of contact, and increased likelihood for faecal contamination of feeding sites, may facilitate disease transfer (Kirkwood et al., 1997; Pennycott et al., 1998b).

WILDLIFE DISEASE SURVEILLANCE

Monitoring the health of free-ranging wildlife populations is required to assess the impact of disease on species' populations and regional biodiversity; to inform

conservation programmes for threatened species adversely affected by disease; to improve individual animal welfare through mitigation of anthropogenic effects; and to detect pathogens potentially transmissible to humans, livestock and companion animals (Daszak et al., 2000; Daszak et al., 2001; Kruse et al., 2004).

Multiple reviews of the schemes for wildlife disease surveillance in the UK since the 1960s have concluded that the status of monitoring was inadequate, in terms of funding and organisation (McDiarmid 1969; Simpson 1988; Osborn et al., 1990). Most recently Sainsbury et al. (2001) concluded that the current schemes are insufficient, with an uncoordinated and fragmented approach, making it plausible that significant disease events may remain undetected with concomitant risks to animal welfare, species conservation, livestock and public health. The VLA's work with wild birds is chiefly concerned with investigation of mass mortality events or targeted surveillance for notifiable disease, principally West Nile virus in passerine species; available resources limit its scope and capacity.

Schemes for wildlife disease investigation and surveillance typically adopt 1 of 2 approaches. Research programmes frequently focus on specific pathogens or assessment of the health of particular species. Targeted research to determine pathogen presence or prevalence may require capture of live animals and sample collection (e.g. blood sample (Gruwell et al. 2000), cloacal swab (Dalessi et al., 2007), conjunctival swabs (Hartup et al., 1999)). These studies have the advantage of defined and controlled methodologies, considering sample size and appropriate randomisation of design. However they are typically cost and labour intensive and frequently require license under relevant legislation (i.e. Animals (Scientific Procedures) Act 1986 in the UK). Considerable expertise in trapping and immobilisation techniques is required and governmental permits for interventions and sampling of protected species must be sought (i.e. permits issued by Natural England under the Wildlife and Countryside Act 1981 in the UK).

Investigations of the causes of wildlife mortality, including infectious disease, rely on post mortem investigation. Communication networks between non-governmental and governmental organisations have been established that encourage the general public to

report observations of wild animal mortality in order to retrieve carcasses for examination. Public reporting of wild bird carcasses has been utilised as a surveillance tool for West Nile virus and Usutu virus (Eidson et al., 2001; Ward et al., 2006; Chvala et al., 2007).

In the UK, schemes to monitor causes of mortality in wild mammals typically focus on species of conservation importance, frequently of large body size and high visibility (e.g. red squirrel (*Sciurus vulgaris*, Sainsbury et al., 2008), otter (*Lutra lutra*, Simpson 1997) and cetaceans (Jepson et al., 2005)). Monitoring of amphibian population health through citizen science projects has taken advantage of the close contact between the general public and wildlife in garden pond habitats (Cunningham et al., 1996).

CITIZEN SCIENCE METHODOLOGIES

Citizen science projects employ a partnership approach between the public and academic communities where large-scale volunteer networks are used to address research questions developed by scientific researchers. Survey methodologies and recording formats are developed to ensure that collected data are of a sufficient quality and quantity for robust analysis (Cooper et al., 2007). This approach facilitates long-term studies, with wide geographical coverage, where it would be impractical and prohibitively expensive for the data collection to be undertaken by scientists or other salaried professionals. A reciprocal benefit has been shown with opportunities for participant education and engagement in scientific 'thinking' within the research context (Trumbull et al., 2000).

Since gardens are accessible to the public year round, citizen science schemes in garden habitats are possible across all seasons which might not be practicable in other environments (Cannon et al., 2005). Appraisal of the importance of private garden habitats for wildlife requires owner involvement for access, by necessity; data is conveniently collected by the homeowner themselves lending research within this habitat to citizen science projects (Cooper et al., 2007).

Project FeederWatch (2009) began in 1987 as the first major citizen science scheme in North America to monitor trends of wild birds within garden habitats from November to March (inclusive) using a combination of online and questionnaire recording formats. The Great Backyard Bird Count (2010) is an annual survey across North America which began in 1997 that provides a snapshot of garden bird species distributions each February.

Citizen science schemes in Great Britain for monitoring wild birds in garden habitats range in their spatial coverage from smaller scale studies e.g. BTO Garden Bird Feeding Survey (GBFS) (Toms 2003), established in 1970 and performed by 250 volunteers, to national schemes such as the BTO's Garden BirdWatch (GBW) (Toms 2003), established in 1995 and performed by c. 16,000 participants and the RSPB's Big Garden BirdWatch established in 1979 and performed by c. 400,000 participants. These schemes vary in terms of their frequency, the RSPB's Big Garden BirdWatch is for a period of a single hour only on a specified weekend each January and the GBFS is restricted to the winter months. The BTO's GBW is the only national scheme that runs year-round with weekly data collected on the number and species of birds reported per site.

Cannon et al. (2005) evaluated use of the BTO's GBW survey results as indicators of general population trends and found the results from garden habitats were meaningful on a national scale for many bird species. North American researchers have similarly found that the Project FeederWatch data can be used to examine spatial and temporal trends in garden use by common bird species and to provide relative indices of species abundance (Wells et al., 1998; LePage et al., 2002).

Citizen science schemes in place to monitor garden bird populations can be extended to incorporate disease surveillance elements. The epidemiology of house finch conjunctivitis in the U.S.A., caused by *Mycoplasma gallisepticum* infection, was investigated through the Project FeederWatch volunteer network. Participants noted evidence of disease in their locality, on the basis of observed clinical signs, and this information was used to track the spread of the condition amongst wild bird populations

(Dhondt et al., 1998). However, for the majority of infectious diseases, no pathognomonic clinical signs or lesions are visible and further pathological investigation is required to reach a firm diagnosis. For example, garden birds affected by salmonellosis, trichomonosis and colibacillosis, infectious diseases diagnosed most frequently in these species in Great Britain, may all display non-specific signs of ill health, including fluffed-up plumage, lethargy and laboured breathing (Kirkwood et al., 1997). This thesis presents the results of the first citizen science study for disease surveillance in wild bird populations that incorporates direct collaboration with wildlife disease investigation centres.

CHAPTER 2: SALMONELLOSIS IN GARDEN BIRDS: TEMPORAL AND SPATIAL LINKS WITH HUMAN INFECTION, 1993 TO 2003

2.1 INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S.* Typhimurium) (Le Minor et al., 1997) is the bacterial agent most commonly responsible for salmonellosis in small passerine species and this condition has been documented internationally since the 1950s, principally at garden bird feeding stations (Hudson et al., 1957; Wilson et al., 1967; Tizard 2004). In Great Britain, the greenfinch (*Carduelis chloris*) and house sparrow (*Passer domesticus*) are the species most frequently reported to be affected by salmonellosis, although other species are also susceptible (Taylor 1968; Kirkwood et al., 1995; Pennycott et al., 1998a). *S.* Typhimurium definitive phage type (DT) 40, DT56 variant(v) and DT160 account for the majority of isolates from garden birds in Great Britain (Penfold et al., 1979; Macdonald et al., 1980; Pennycott et al., 2006). Garden bird *S.* Typhimurium phage types are thought to have a narrow host range and may be highly host-adapted (Rabsch et al., 2002) and clonal in some instances (Alley et al., 2002). These findings support the hypothesis that wild passerine populations act as the primary reservoir of these *S.* Typhimurium phage types.

In general, salmonellosis caused by infection with any one of a range of over 2500 existing *S. enterica* serovars classified by the Kauffmann-White scheme can result in serious disease in humans with infant, geriatric and immunosuppressed individuals being particularly vulnerable (Gordon 2008). The role of wild bird populations as reservoirs of zoonotic pathogens is an important current research topic (Tsiodras et al., 2008).

Garden bird phage types of *S.* Typhimurium have zoonotic potential through the faeco-oral route of transmission. Genotypically identical strains of *S.* Typhimurium have been found in sympatric populations of wild birds and humans (Hauser et al., 2009). Where epidemic mortality due to salmonellosis has occurred in garden birds, for example in Sweden (Tauni et al., 2000) and New Zealand (Alley et al., 2002),

large numbers of humans have also become infected resulting in clinical symptoms. The extent to which passerine populations act as a source of human infection in Great Britain is unknown.

This chapter describes the occurrence of salmonellosis in garden birds in England and Wales from 1993 to 2003 inclusive. Trends in the temporal and geographical occurrence of salmonellosis, and in the *S.* Typhimurium phage types involved, are reviewed: the age, sex and species composition of affected birds are also described.

The regional distribution, temporal and seasonal trends of *S.* Typhimurium DT40, DT56(v) and DT160 infection in humans from England and Wales between 1993 and 2003 are compared with the salmonellosis incidents in garden bird species for the same time period. The age of patients with salmonella infection is also summarised. The hypothesis that an epidemiological link between salmonellosis in human and garden bird populations exists is assessed, including the extent to which garden birds might act as a source of human infection and the likely routes of transmission that might occur are discussed in the light of the available data.

2.2 MATERIALS AND METHODS

Garden bird mortality reports and acquisition of carcasses

Since 1993, opportunistic reports of garden bird mortality incidents in England and Wales were solicited from members of the public through a passive surveillance network, including the British Trust for Ornithology (BTO), the Royal Society for the Protection of Birds, the Universities Federation for Animal Welfare and the Institute of Zoology.

A mortality incident was classified as one or more dead birds found at a particular site within a 30-day period. Where available, carcasses were submitted to the Institute of Zoology for post mortem examination (PME) following a standardised protocol. Details of the date found, geographical location and clinical signs observed were recorded.

Garden bird post mortem examinations

Each submitted carcass was assigned a unique PME reference code. Birds were refrigerated at 4 °C and examined fresh within 48 hours of submission where possible, or were frozen at -20 °C on submission and examined at a later date. The species, age, sex and body mass were recorded for each bird examined. Birds were classed as juveniles until the post-juvenile body moult was complete. First-year birds beyond their post-juvenile moult and adult birds were not differentiated. Sex was assigned based on gonadal inspection and/ or plumage characteristics. Qualitative body condition scores ('emaciated', 'thin', 'normal', 'fat') were assigned based on visual inspection of pectoral muscle mass and fat deposits. Systematic external and internal examinations of body systems were performed and any gross lesions described. Where indicated, and where the degree of carcass decomposition permitted, samples were taken for microbiological, parasitological, histopathological and toxicological investigations. An archive of frozen and of formalin-fixed samples was created to facilitate retrospective analysis. Cause of death (COD) categories were assigned in each case based on a review of all findings; these comprised: 'infectious disease', 'predation', 'other trauma', 'other' and 'not established'.

The liver, small intestine and/or crop/oesophagus, in addition to any lesions found, were routinely sampled aseptically and examined for the presence of pathogenic bacteria using a standard protocol. Briefly, tissue samples were plated directly onto (1.) Colombia blood agar supplemented with 5% horse blood (QCM laboratories, London, UK) in triplicate, and incubated under each of aerobic, anaerobic and microaerophilic conditions; (2.) Xylose-Lysine Deoxycholate (XLD) agar (QCM laboratories) and incubated under aerobic conditions; (3.) Chocolate blood agar (QCM laboratories) and incubated under CO_2 conditions: and (4.) immersed in selenite Salmonella-selective enrichment broth (QCM laboratories) under aerobic conditions for 24 hours followed by subculture onto XLD agar aerobically. All samples were incubated at 37 °C with inspection of the agar plates at 24, 48 and 120 hours post-inoculation. Bacterial isolates were identified using colony and organism morphology, Gram's staining and biochemical properties, which were determined using the analytical profile index (API) biochemical test strip method (API-BioMerieux, Marcy l'Etoile, France).

Slide agglutination tests were performed for the identification of suspected *Salmonella* spp. isolates using poly-O antisera (Pro-lab diagnostics, Neston, UK). Salmonella isolates were placed onto microbank beads (Pro-lab diagnostics) and stored at both –25 °C and –70 °C. Batches of isolates were later submitted to the UK Veterinary Laboratories Agency or to the Salmonella Reference Unit (SRU) of the Health Protection Agency (HPA) (England, Wales and Northern Ireland) for biotyping (serotype and phage type) according to standardised international protocols (Anderson et al., 1977).

In cases where a *Salmonella* sp. was isolated from a lesion, or lesions, characteristic for salmonellosis in the absence of any other obvious COD, a diagnosis of salmonellosis was made.

Cases of human infection with *S.* Typhimurium DT40, DT56(v) and DT160

The SRU, HPA, provided details of all *S.* Typhimurium DT40, DT56(v) and DT160 isolates from humans that were confirmed between 1993 and 2003 from England and Wales. Salmonella isolates from clinical cases were submitted by hospitals and other medical referral facilities to the HPA for serotyping and phage typing (Anderson et al., 1977). Salmonella isolates from human cases with a recent history of international travel were excluded from this study. The human salmonella isolates selected were matched to the antibiotic sensitivity profiles of the passerine *S.* Typhimurium isolates. No information on the clinical signs at presentation was available although enteric infection with gastroenteritis was assumed to be the major presenting complaint. The month when the isolates were submitted for typing was summarised by season; winter (Dec-Feb), spring (March-May), summer (June-Aug), autumn (Sept-Nov). Affected individuals were categorised by age as infant (0-5 years), child (>5-16 years), adult (>16 -65 years) and geriatric (>65 years). The home town for each human case was not available; instead the location of the referring medical facility, summarised by National Government Office Region (GOR), was used as an approximate guide to examine the case distribution.

Data analyses

Comparisons were made between the frequencies of salmonellosis cases by species, and by season for the greenfinch and house sparrow respectively, using the Pearson's

chi-square test (Crawley 2002). Where the frequencies involved were small, analyses were conducted using the Fisher's exact test (Crawley 2002). The binomial test (Crawley 2002) was used to compare the proportion of the salmonellosis cases which were male, for the greenfinch and house sparrow respectively, with a population with a sex ratio of unity (i.e. 50% male, 50% female). The proportion of males in the control group for each species (i.e. birds that died as a result of predation or 'other trauma' with no evidence of infectious disease) was also compared with a population with a sex ratio of unity. Data deficient cases were excluded from the analyses.

Comparisons were made between the frequencies of human cases of salmonella infection for each of the phage types by season and age group using the Pearson chi-square test (Crawley 2002). The binomial test (Crawley 2002) was used to compare the proportion of human cases of salmonella infection that occurred in the modal season for each of the phage types with the proportional sum for the other phage types.

The Spearman rank correlation for non-parametric data was used to assess the association between the spatial distribution of human cases and garden bird incidents for each *S.* Typhimurium phage type by GOR. The number of human infections was expressed as a percentage of the regional human population (based on the 2001 census data) in order to correct, as far as possible, for variation in the human population density across the GORs.

Statistical analyses were performed using SPSS 17.0 for Windows (SPSS inc., Chicago, U.S.A.) and R-CRAN (http://www.R-project.org). Spatial data were presented using ArcView 3.0 geographical information system (GIS) software (Environmental Systems Research Institute GIS and Mapping Software, California, U.S.A.).

2.3 RESULTS

Garden bird species

During the period 1993-2003 inclusive, 698 garden birds were examined post mortem. These comprised 656 from the order Passeriformes (37 species, 14 families); 27 Columbiformes (3 species, 1 family); 8 Piciformes (2 species, 1 family); 5 Accipitriformes (1 species) and single birds from the orders Cuculiformes and Strigiformes. Salmonellosis was confirmed as the COD of 157 birds from 118 incidents (112 sites), but in only 7 of the 45 species examined. Data for each of the 20 most commonly reported garden bird species, as defined by the BTO's Garden BirdWatch (GBW) scheme (BTO 2010), in addition to all other species (n = 2) in which salmonellosis was diagnosed, is summarised in Table 2.1.

Table 2.1: Summary data for the number of birds examined post mortem during the study. The list includes each of the most common 20 British bird species, as listed by the BTO Garden BirdWatch (GBW) scheme, in addition to all species in which cases of salmonellosis were diagnosed. Species order is presented by bird family in alphabetical order.

[a]GBW ranking taken from Quarter 1 (Jan – March) 2003
[b]Cases in which a *Salmonella* sp. was isolated but the COD was not established.

Garden BirdWatch ranking[a]	Species	Latin name	Order/ Family	No of birds	Male	Female	Undetermined	No of incidents involving species	No of salmonellosis cases	Male	Female	Undetermined	No of salmonellosis incidents involving species
7	Collared dove	*Streptopelia decaoacto*	Columbiformes Columbidae	16	7	7	2	15	0	0	0	0	0
11	Wood pigeon	*Columba palumbus*	Columbiformes Columbidae	3	1	2	0	3	0	0	0	0	0
18	Carrion crow	*Corvus corone*	Passeriformes Corvidae	3	2	0	1	3	0	0	0	0	0
16	Long tailed tit	*Aegithalos caudatus*	Passeriformes Aegithalidae	4	1	1	2	4	0	0	0	0	0
19	Jackdaw	*Corvus monedula*	Passeriformes Corvidae	0	0	0	0	0	0	0	0	0	0
12	Magpie	*Pica pica*	Passeriformes Corvidae	4	1	1	2	4	0	0	0	0	0
29	Bullfinch	*Pyrrhula pyrrhula*	Passeriformes Fringillidae	5	2	3	0	5	2	0	2	0	2
5	Chaffinch	*Fringilla coelebs*	Passeriformes Fringillidae	18	10	7	1	16	6 (1[b])	4 (0[b])	2 (1[b])	0 (0[b])	5 (1[b])
17	Goldfinch	*Carduelis carduelis*	Passeriformes Fringillidae	2	1	0	1	2	1	1	0	0	1
8	Greenfinch	*Carduelis chloris*	Passeriformes Fringillidae	195	103	57	35	160	118	64	37	17	94

Garden BirdWatch ranking[a]	Species	Latin name	Order/ Family	No of birds	Male	Female	Undetermined	No of incidents involving species	No of salmonellosis cases	Male	Female	Undetermined	No of salmonellosis incidents involving species
21	Siskin	*Carduelis spinus*	Passeriformes Fringillidae	25	18	5	2	10	1 (1[b])	1 (1[b])	0 (0[b])	0 (0[b])	1 (1[b])
2	Blue tit	*Cyanistes caeruleus*	Passeriformes Paridae	63	32	10	21	38	1	0	1	0	1
13	Coal tit	*Periparus ater*	Passeriformes Paridae	6	0	4	2	6	0	0	0	0	0
6	Great tit	*Parus major*	Passeriformes Paridae	6	3	1	2	6	0	0	0	0	0
9	House sparrow	*Passer domesticus*	Passeriformes Passeridae	99	35	34	30	79	28 (2[b])	12 (1[b])	13 (0[b])	3 (1[b])	22 (2[b])
4	Dunnock	*Prunella modularis*	Passeriformes Prunellidae	10	3	1	6	8	0	0	0	0	0
10	Starling	*Sturnus vulgaris*	Passeriformes Sturnidae	16	5	3	8	9	0	0	0	0	0
14	Wren	*Troglodytes troglodytes*	Passeriformes Troglodytidae	1	0	0	1	1	0	0	0	0	0
1	Blackbird	*Turdus merula*	Passeriformes Turdidae	66	35	11	20	51	0	0	0	0	0
3	Robin	*Erithacus rubecula*	Passeriformes Turdidae	8	4	3	1	8	0	0	0	0	0
15	Song thrush	*Turdus philomelos*	Passeriformes Turdidae	86	51	17	18	76	0 (1[b])	0 (1[b])	0 (0[b])	0 (0[b])	(1[b])
20	Great Spotted woodpecker	*Dendrocopus major*	Piciformes Picidae	3	1	1	1	2	0	0	0	0	0
	TOTAL			639	315	168	156	506	157	82	55	20	126

Table 2.2: Number of mortality incidents and number of birds of each species from which each phage type of *S.* Typhimurium was isolated

Family		Fringillidae					Paridae	Passeridae	
Phage type	No. of mortality incidents (%)	Bullfinch *Pyrrhula pyrrhula*	Chaffinch *Fringilla coelebs*	Goldfinch *Carduelis carduelis*	Greenfinch *Carduelis chloris*	Siskin *Carduelis spinus*	Blue tit *Cyanistes caeruleus*	House sparrow *Passer domesticus*	Total No. of birds (%)
1	2 (1.7)	0	0	0	2	0	0	0	2 (1.3)
37	1 (0.8)	0	0	0	0	0	0	1	1 (0.6)
40	60 (50.8)	1	2	1	61	1	0	8	74 (47.2)
56 var	28 (23.7)	0	2	0	33	0	0	5	40 (25.5)
87 var	1 (0.8)	0	0	0	1	0	0	0	1 (0.6)
120	1 (0.8)	0	0	0	1	0	0	0	1 (0.6)
129	1 (0.8)	0	0	0	0	0	1	0	1 (0.6)
160	7 (5.9)	0	0	0	3	0	0	6	9 (5.7)
193	1 (0.8)	0	1	0	0	0	0	0	1 (0.6)
U313	1 (0.8)	0	0	0	0	0	0	3	3 (1.9)
RDNC	3 (2.5)	0	0	0	3	0	0	0	3 (1.9)
ND	12 (10.2)	1	1	0	14	0	0	5	21 (13.4)
Total	118	2	6	1	118	1	1	28	157

RDNC= Reacts does not confirm, ND= Isolate not phage typed

Salmonella sp. were isolated from a further 5 birds (4 species, 5 incidents) where the findings did not fulfil the criteria for salmonellosis as a COD, or where PME was inconclusive. In these cases, also summarised in Table 2.1, the bacterium may have been a cause of clinical disease or asymptomatic intestinal carriage but differentiation between these possibilities was not possible. Two of the isolates from 1chaffinch and 1 house sparrow, submitted from different sites, were *S.* Typhimurium DT40 and the isolate from a song thrush was identified as *Salmonella arizonae* (on the basis of API profile), although no further biotyping was performed. Two further isolates from 1 house sparrow and 1 siskin, submitted from different sites, were not biotyped. These 5 birds and their isolates were excluded from all data analyses.

Greenfinch and house sparrow were the species most commonly diagnosed with salmonellosis, both in absolute numbers of cases and in numbers of incidents: 61% (118/195) of greenfinches submitted and 28% (28/99) of house sparrows submitted were diagnosed with salmonellosis. Submitted greenfinches were, therefore, more likely to have died of salmonellosis than submitted house sparrows (X^2=27.28, df=1, P<0.001). Of the species where the sample size of carcasses examined was high enough ($\geq$ 5) to allow statistical comparison, the only species submitted which was as likely to have died with salmonellosis as a greenfinch was the bullfinch (Fisher's exact test P=0.39).

Single birds were submitted for examination from 89 incidents whilst multiple carcasses were examined post mortem from the remainder (29 incidents, 23 sites). From the latter group, mortality of multiple birds over a 30-day period, equating to a single mortality incident, was recorded at 18 sites, while 2 salmonellosis mortality incidents within the same year occurred at 2 sites (4 incidents) and mortality incidents in consecutive years were recorded at 3 sites (7 incidents). Salmonellosis was confirmed in multiple birds from the same mortality incident at 23 sites: the species involved were greenfinches only (11 sites), house sparrows only (1 site), greenfinches and house sparrows together (8 sites), and greenfinches and chaffinches together (3 sites).

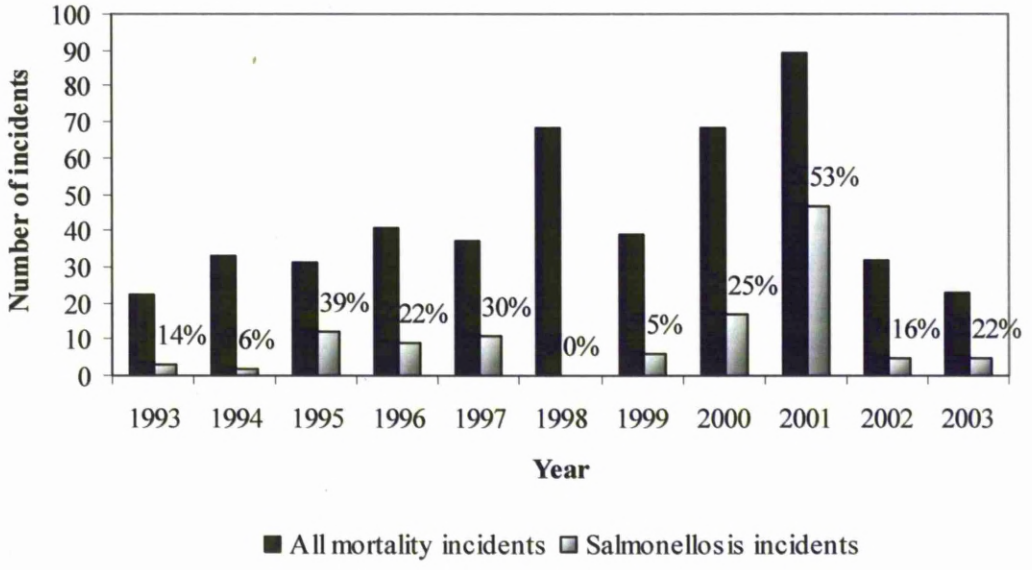
Serotype and phage type in garden birds

One hundred and fifty seven salmonella isolates were cultured during the course of this study. Of these 145 were fully serotyped and all were identified as *S.* Typhimurium. Phage typing was performed on 136 of these isolates and details of the phage type, and bird species from which they were isolated, are presented in Table 2.2. Ten phage types were identified and most were isolated from only 1 bird or 1 mortality incident. Ninety percent of the *S.* Typhimurium isolates phage typed belonged to 3 phage types: 54% DT40 (60 mortality incidents, 74 birds), 29% DT56(v) (28 mortality incidents, 40 birds), and 7% DT160 (7 mortality incidents, 9 birds). *S.* Typhimurium provisional phage type U313 was isolated from 3 house sparrows (from a single mortality incident). This phage type was first recognised in 2000 and will be given a definitive phage type following further work by the SRU of the HPA. Three *S.* Typhimurium isolates from 3 greenfinches (from 3 mortality incidents) reacted with typing phages but did not confirm to any of the recognised typing patterns: they were therefore classified as 'reacts but does not conform' (RDNC). Each mortality incident involved only 1 *S.* Typhimurium phage type or RDNC isolate.

Annual trends in garden birds

Salmonellosis outbreaks were identified in each year of the 11-year study period with the single exception of 1998. Variation was seen in both the number of garden bird mortality incidents reported each year and in the annual percentage of incidents diagnosed as being due to salmonellosis (Figure 2.1). There was an apparent peak in salmonellosis incidents in 2001, when this disease accounted for 53% (47/89) of the avian mortality incidents investigated.

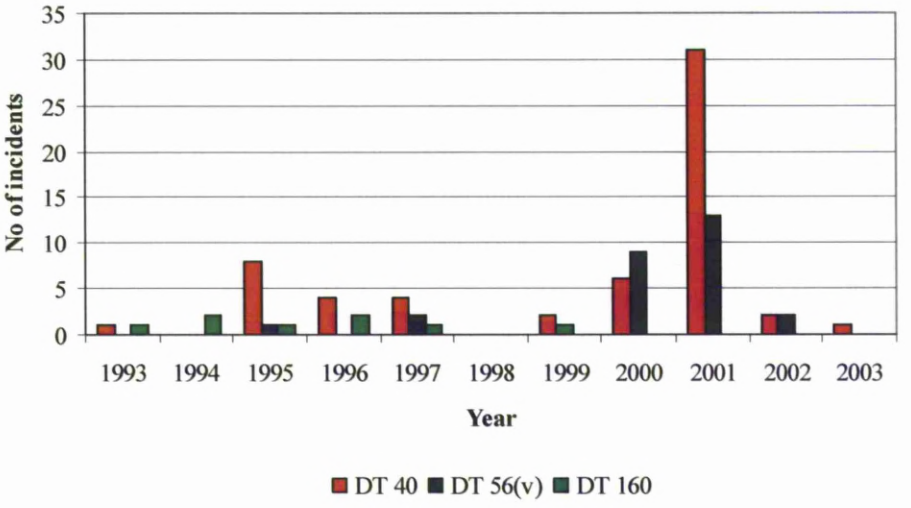
Figure 2.1: Total number of avian mortality incidents investigated per annum and total number attributed to salmonellosis by year, 1993 – 2003.
The percentage of mortality incidents per annum due to salmonellosis is shown.



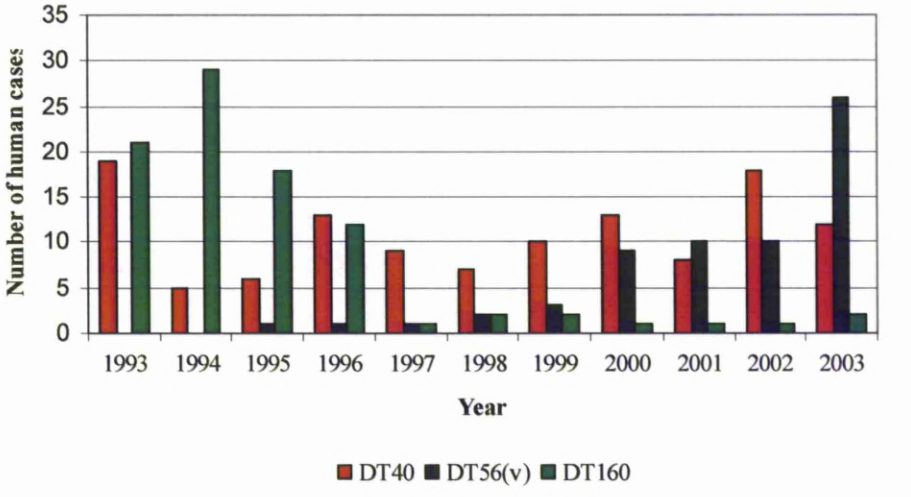
Over the course of the 11-year study period, the prevalences of the isolated *S.* Typhimurium phage types changed (Figure 2.2). *S.* Typhimurium DT160 was only isolated during the first 5-year period of the study (1993-1997) whilst. *S.* Typhimurium DT56(v) was found sporadically until 2000 and 2001, when it increased considerably in prevalence. The incidence of both phage types decreased markedly after 2001 (to zero for DT56(v) in 2003). Throughout the study, however, *S.* Typhimurium DT40 was the most common phage type overall, with an apparent peak in prevalence in 2001 (Figure 2.2a).

Figure 2.2: (a) Number of salmonellosis incidents in garden birds caused by *S.* Typhimurium DT40, DT56(v) and DT160, (b) Number of human cases with *S.* Typhimurium DT40, DT56(v) and DT160 infection by year; 1993 – 2003.

(a)



(b)



Annual trends in humans

A total of 273 garden bird *S.* Typhimurium isolates, comprising 120 DT40, 63 DT56(v) and 90 DT160 phage types were confirmed in humans between 1993 and 2003. Annual trends in the human infection data closely matched those observed in the garden bird salmonellosis incidents for each of the common *S.* Typhimurium phage types (Figure 2.2b) suggesting a possible epidemiological link. DT40 isolates

were found throughout the study period, DT160 isolates were observed chiefly between 1993 and 1996 reducing to low numbers in later years and DT56(v) cases were identified with increasing frequency from 2000. The number of garden bird salmonellosis incidents due to DT160 correlated positively (Pearson correlation coefficient r=0.788, P=0.004) with the number of human cases of infection caused by this phage type across the study period; no similar significant association was found for DT40 (P>0.05) or DT56(v) (P>0.05).

Geographical distribution in garden birds

Greenfinch and house sparrow carcasses were submitted for PME from most regions of England and Wales (Figure 2.3). There was an annually recurring trend in the geographical distribution of cases of salmonellosis, with cases widespread across the English Midlands, the English/Welsh border region and southern England (Figure 2.4). Whilst this pattern generally reflected the geographic pattern of carcass submissions, there was a notable absence of the disease across central England and East Anglia; an area from which numerous carcass submissions were received.

Figure 2.3: The distribution of greenfinches and house sparrows examined post mortem, 1993-2003.
Blue circles indicate greenfinches and red circles indicate house sparrows.

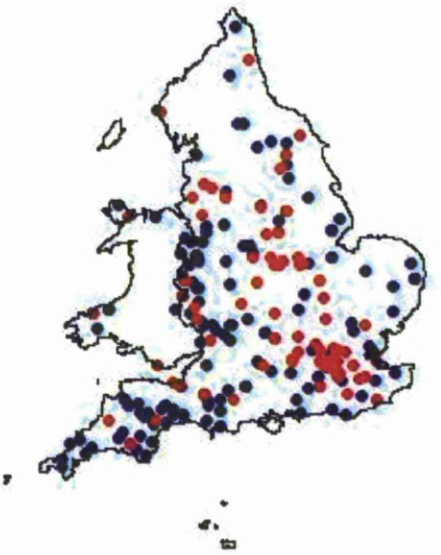
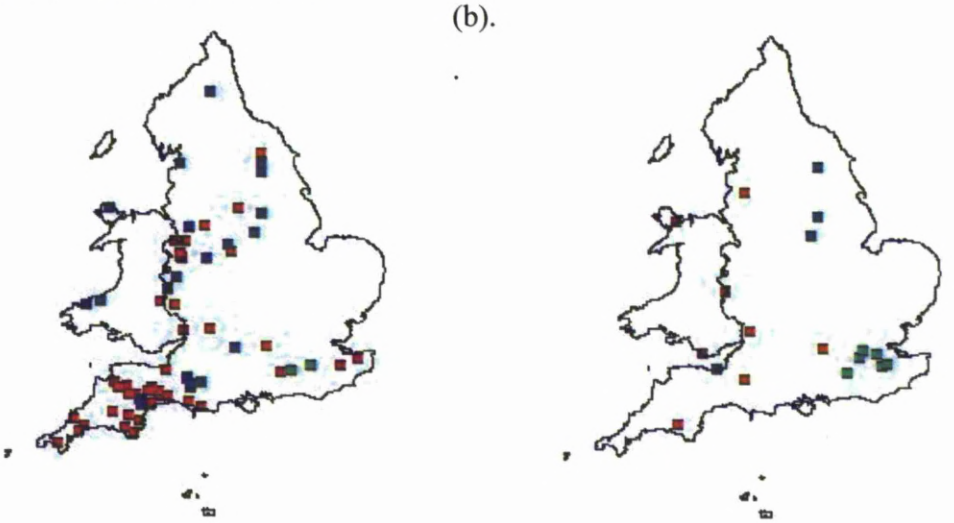


Figure 2.4: The distribution of *S.* Typhimurium phage types for (a) greenfinches and (b) house sparrows, 1993-2003. Red squares indicate DT56(v); blue squares indicate DT40; green squares indicate DT160.

(a) (b).

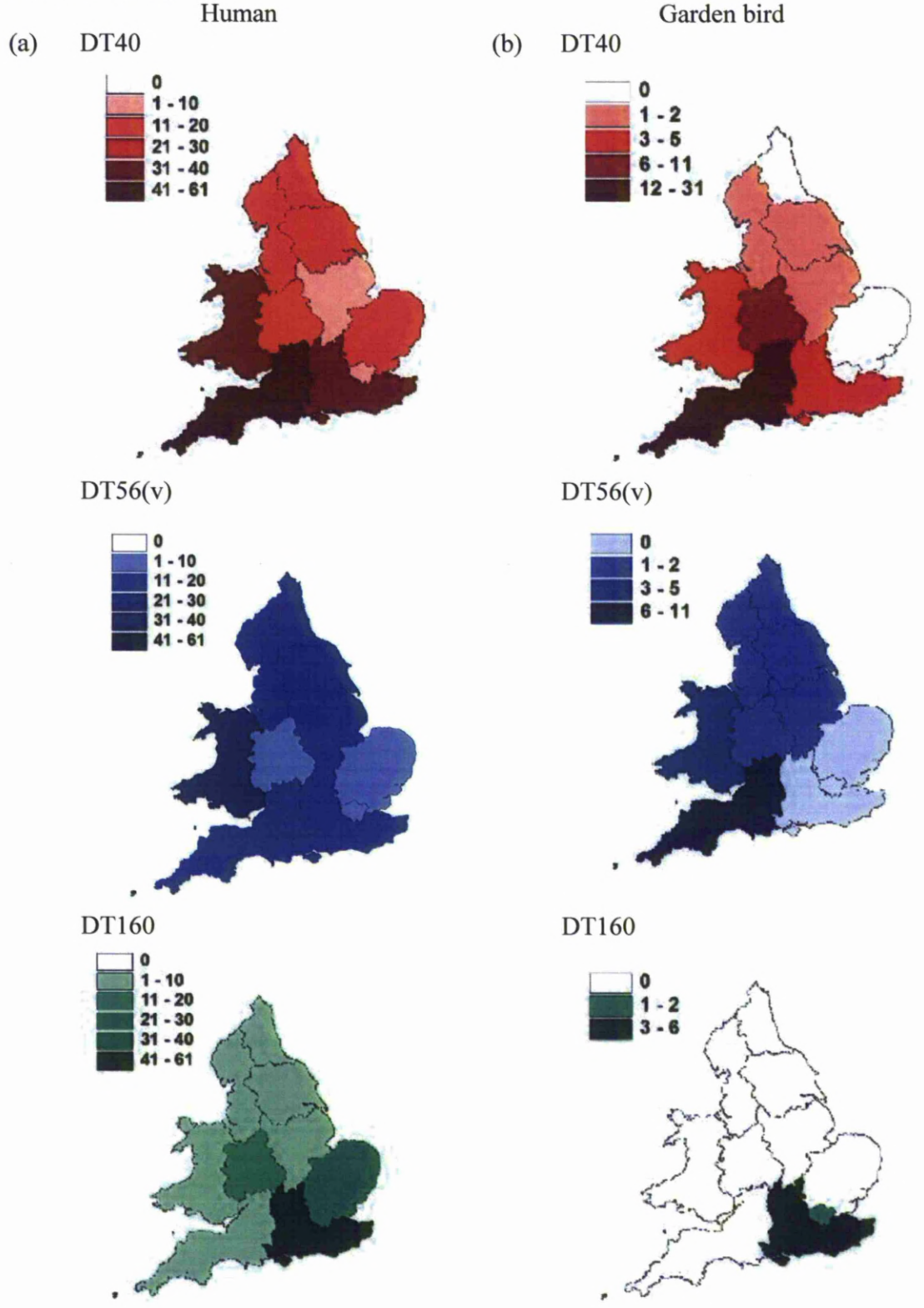


Geographical trends were noted also for the occurrence of salmonellosis due to specific *S.* Typhimurium phage types. Mortality due to phage types DT40 and DT56(v) tended to occur in western Britain, whilst mortality incidents due to DT160 were clustered within south-east England (Figure 2.4). Geographical patterns of salmonellosis and phage types were similar for both house sparrows and greenfinches. The number of birds of other species diagnosed with salmonellosis was too small for meaningful examination of incident distribution but similar trends were seen as for the phage types isolated from greenfinches and house sparrows.

Geographical distribution in humans

The spatial distribution of *S.* Typhimurium DT40, DT56(v) and DT160 human cases of infection was compared with that of the garden bird salmonellosis incidents using the national GORs (Figure 2.5). A significant positive correlation was found between the datasets for *S.* Typhimurium DT40 (Spearman rank correlation coefficient r=0.821, P=0.04), DT56(v) (r=0.725, P=0.018) and DT160 (r=0.664, P=0.036).

Figure 2.5: Distribution of *S.* Typhimurium DT40, DT56(v) and DT160 incidents, 1993-2003 (a) Human data expressed as number of cases per 10,000,000 people by region, according to the 2001 census data. (b) Garden bird data expressed as total number of incidents by region.



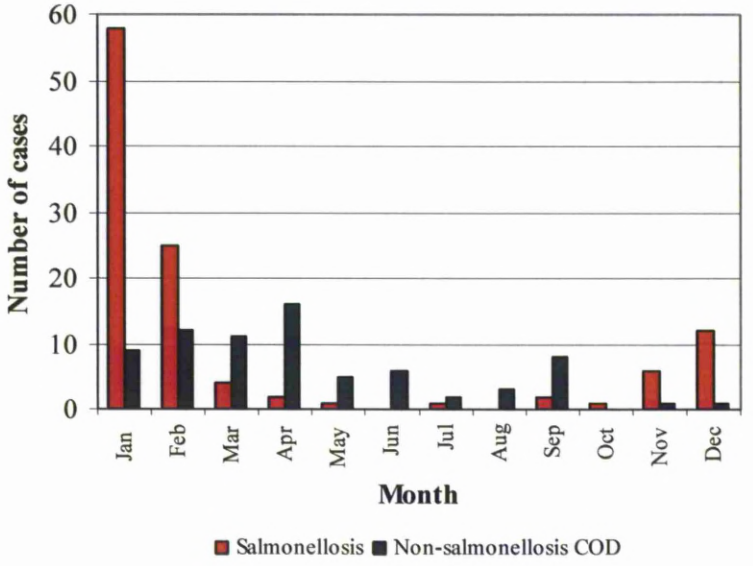
Seasonal trends in garden birds

For each year of the study, salmonellosis incidents in garden birds showed a marked seasonal trend, with peak occurrence during the winter months, December-February (91/118 - 77% incidents). A similar seasonal trend was seen for the isolation of each of the common *S.* Typhimurium phage types; 77% (46/60) of DT40 incidents and 86% (24/28) of DT56(v) incidents occurred from December to February inclusive.

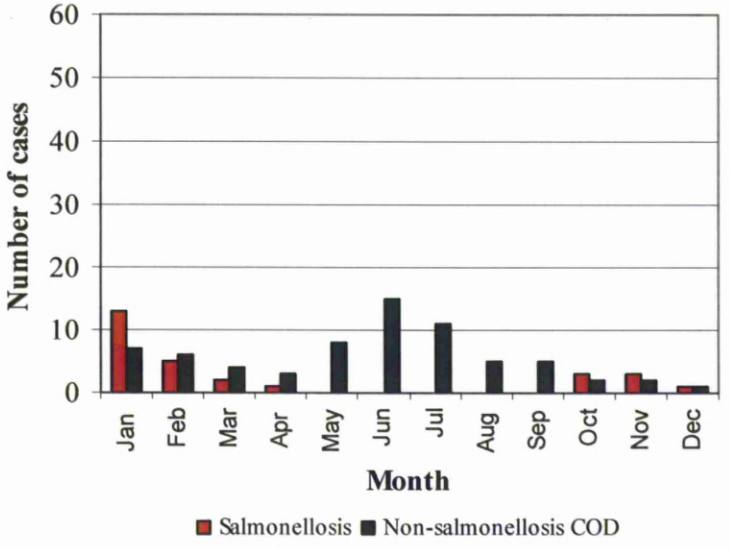
Dead greenfinches and house sparrows were submitted during each month of the year (Figure 2.6): January was the peak month both for the number of mortality incidents due to salmonellosis and for the number of birds found dead with this disease. From 1st September to 30th April inclusive, 116 of 177 greenfinches submitted were diagnosed with salmonellosis compared with 28 of 60 house sparrows, therefore submitted greenfinches were more likely to have salmonellosis than submitted house sparrows (X^2=6.69, df=1, P=0.01) during this period., Only 9% (18 of 195) of submitted greenfinch carcasses were found in the summer months (1st May to 31st August, inclusive), of which 2 (from separate incidents) were confirmed as having died of salmonellosis (Figure 2.6a). In contrast, 39% (39 of 99) of house sparrows submitted were found dead from 1st May to 31st August (inclusive), but no cases of salmonellosis were diagnosed in this species during this period (Figure 2.6b). However, the difference in frequency of salmonellosis cases in submitted greenfinches compared with house sparrows in the summer was not significant (Fisher's exact test P>0.05).

Figure 2.6: Monthly distribution of (a) greenfinch submissions and (b) house sparrow submissions by COD category, 1993 – 2003.

(a)



(b)



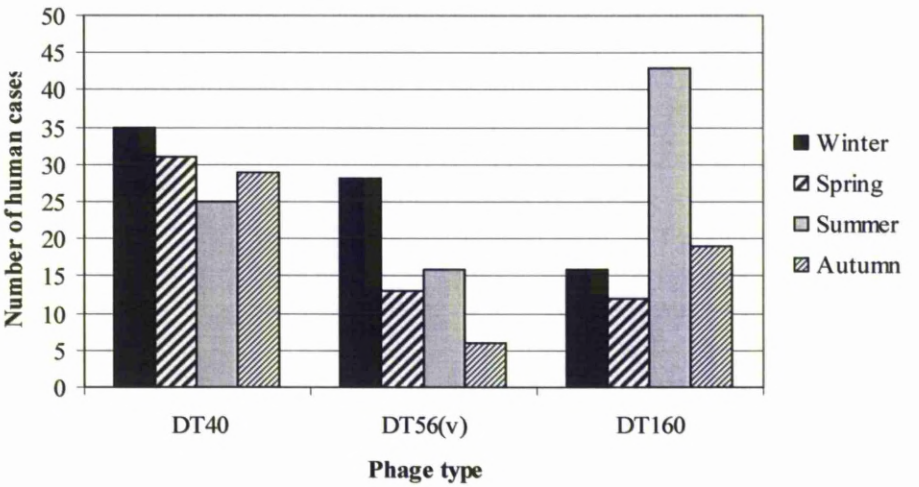
Seasonal trends in humans

The seasonal breakdown of human cases from which *S.* Typhimurium garden bird phage types were isolated comprised 29% in winter (79/273), 20% in spring (56/273), 31% in summer (84/273) and 20% in autumn (54/273). There was a significant difference among the seasonal pattern of human cases between the 3 *S.* Typhimurium phage types (X^2=30.53, df=6, P<0.000) (Figure 2.7). The modal season

for *S.* Typhimurium DT40 was winter although there was least variation in the number of human cases between seasons for this phage type. The percentage of human cases in winter with *S.* Typhimurium DT40 (29.2%) was similar to the proportion of human cases due to *S.* Typhimurium DT56(v) and DT160 combined (28.8%) for that season (binomial test of proportions P>0.05). The modal season for *S* Typhimurium DT56(v) was also winter. The proportion of human cases in winter with *S.* Typhimurium DT56(v) was significantly greater than the proportion of human cases due to *S.* Typhimurium DT40 and DT160 combined for that season (binomial test of proportions X^2=8.62, df=1, P=0.003).

In contrast, the modal season for *S.* Typhimurium DT160 was summer. The proportion of human cases in summer with *S.* Typhimurium DT160 was significantly greater than the proportion of human cases due to *S.* Typhimurium DT40 and DT56(v) combined for that season (binomial test of proportions X^2=17.06, df=1, P<0.0001).

Figure 2.7: Number of human cases with *S.* Typhimurium DT40, DT56(v) and DT160 infection by season, 1993 to 2003 inclusive. Winter (December – February); spring (March – May); summer (June – August); autumn (September – November).



Case demography in garden birds

All cases of salmonellosis were in adult birds or in first year birds which had progressed beyond their post-juvenile body moult. A greater number of salmonellosis cases was confirmed in male greenfinches than females; the proportion of males in

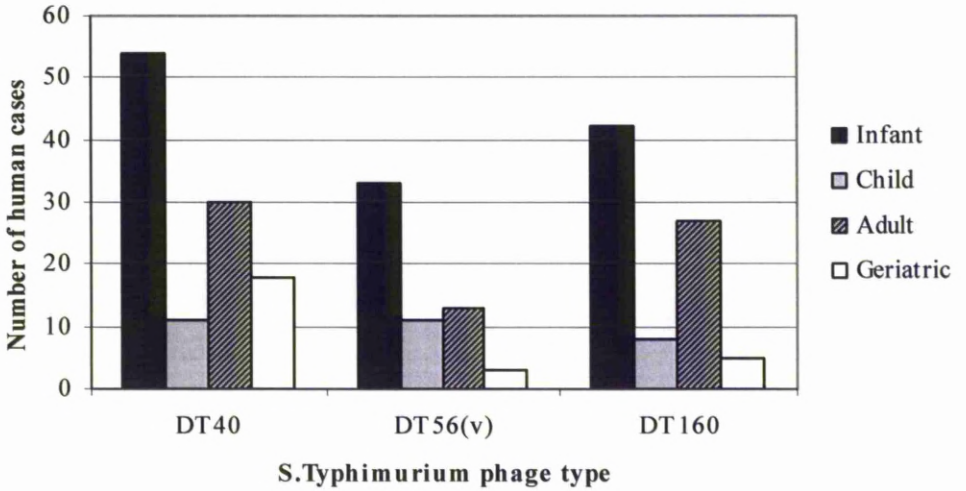
the salmonellosis cases was significantly greater than that for a theoretical population with a sex ratio of unity (64 males, 37 females; X^2 =6.79, df=1, P =0.009). The sex ratio of greenfinches which had died as a result of predation or 'other trauma', with no evidence of concurrent infectious disease, did not differ from unity (4 males, 4 females), which may support a real sex bias with males over-represented in the salmonellosis group. In contrast, neither the proportion of male house sparrows which died of salmonellosis (12 males, 13 females; X^2 =0.00, df=1, P>0.05), nor the proportion of male house sparrows which died of predation or 'other trauma', with no evidence of concurrent infectious disease, (6 males, 7 females; X^2 =0.00, df=1, P> 0.05), differed from that of a population with a sex ratio of unity.

Most greenfinches (103 of 110) and house sparrows (22 of 27) which died of salmonellosis, and which were condition scored, were either emaciated or thin. In contrast, the body condition values for adult greenfinches and house sparrows that died of trauma with no evidence of infectious disease were normal or fat for 5 of 7 greenfinches and for all 6 house sparrows in this COD category. Of the salmonellosis cases, where accurate body weights were available, average values were 21.9 g +/- 0.2 SEM. (n=99) and 21.2 g +/- 0.5 SEM. (n=25) for greenfinches and house sparrows respectively. For the trauma cases, average values for body weight were 23.6 +/- 1.2 SEM. (n=6) and 26.9 +/- 2.1 SEM (n=5) for greenfinches and house sparrows respectively.

Case demography in humans

The demographic breakdown of human cases from which *S.* Typhimurium garden bird phage types DT40, DT56(v) and DT160 were isolated comprised 47% infants (129/273), 11% children (30/273), 26% adults (70/273), 10% geriatric (26/273) and 7% data deficient (18/273).

Figure 2.8: Demographic composition of the human cases with *S.* Typhimurium DT40, DT56(v) and DT160 infection, 1993 to 2003.



There was no significant difference in the demographic breakdown of human cases among the 3 *S.* Typhimurium phage types (X^2=11.53, df=6, P>0.05) (Figure 2.8).

In summary, greenfinches and house sparrows were the species most frequently affected by salmonellosis, principally during the winter months. Similar annual and spatial trends occurred in the passerine and human infections with these *S.* Typhimurium phage types, from 1993 to 2003, supporting a possible epidemiological link.

2.4 DISCUSSION

Garden bird salmonellosis: species affected and case demography

The greenfinch and the house sparrow were the 2 most frequently submitted garden bird species found dead by the British public in this period of study. This is perhaps not surprising as the greenfinch and house sparrow are among the 'top ten' bird species reported in British gardens according to the national GBW scheme, although the greenfinch and the house sparrow, the 8th and 9th most common garden bird species respectively in 2003, were submitted in much larger numbers than higher ranking species of garden bird (Table 2.1). Salmonellosis was the most common

infectious COD of garden birds submitted for PME from 1993-2003 and the greenfinch and the house sparrow were the species most frequently affected. Large numbers of PMEs were conducted on other common garden birds, including 'top ten' species, such as the blackbird, robin, dunnock, great tit, collared dove and song thrush, but there was no evidence of salmonellosis in any of these species.

GBW participants reported seasonal variation in the reporting rates (percentage of gardens with the species present) of the greenfinch and chaffinch; the chaffinch had greater average reporting rates in the autumn and winter seasons (BTO 2010). However, the numbers of incidents and cases of salmonellosis in greenfinches in winter far outweighed those in chaffinches, indicating a real predisposition of the greenfinch to this disease in Britain. This concurs with other reports which identified the greenfinch as the species most frequently affected by salmonellosis in Great Britain (Wilson et al., 1967; Routh et al., 1995; Pennycott et al., 1998a; Grant et al., 2007).

Studies of passerine birds elsewhere have found a predisposition of finches to salmonellosis. In Norway, Refsum et al. (2003) examined 179 passerines post mortem and found 118 of 123 salmonellosis cases to be in finches, 4 cases in other (Paridae) species and a single case in a tree sparrow (*Passer montanus*). The bullfinch, the only species submitted in this study which was as likely to have died with salmonellosis as the greenfinch, was the species found to be most frequently affected by salmonellosis in Norway (Refsum et al., 2003). A review of avian salmonellosis in the U.S.A. found mortality in 98 species from 12 orders with passerines being the most frequently affected group: 21.5% of all passerine mortality events were attributed to salmonellosis (Hall et al., 2008). Of these, 47 (36.4%) events involved only fringillid species and 28 (21.7%) involved fringillid and other passerine species.

Observed variation in disease and infection rates amongst species may be due to intrinsic variation in species susceptibility or due to differential rates of exposure related to species ecology, such as social interactions, dominance hierarchies or feeding behaviour (Refsum et al., 2003; Hall et al., 2008). For example, the greenfinch and house sparrow are gregarious and flock-feeding, particularly during

the winter months (Taylor 1968). Congregation in groups is likely to favour horizontal transmission of disease through high contact rates and greater opportunities for faecal-oral transmission. Feeding stations on the ground or on bird tables will increase the likelihood of faecal contamination of foraging areas, particularly where a high intensity of provisioning is practised (Kirkwood 1998; Kirkwood et al., 1998).

The duration of foraging episodes also might influence infection risk. Finches tend to feed at ground or table feeding stations for prolonged periods of time, thereby increasing their likelihood of coming into contact with contaminated material (Daoust et al., 2000). Also, species such as finches that are commonly affected by salmonellosis share a granivorous diet and tend to manipulate seed in their bill whereas some other granivorous species, such as tits, in which salmonellosis is rarely found, tend to handle seeds with their feet (Toms 2003). The potential for faecal-oral transmission from contaminated seed husks might, therefore, be greater for finches (Refsum et al., 2003). Although salmonellosis has been recorded in insectivorous species, such as the blackbird (Alley et al., 2002), the dunnock and the green woodpecker (*Picis viridis*) (Macdonald et al., 1969), these cases appear to be rare, which is supported by the current study.

For incidents of salmonellosis that involved multiple species, the greenfinch was affected in all except one (in which house sparrows and one other species were affected). These findings support the hypothesis proposed by Pennycott et al. (1998a) that the greenfinch plays a central role in the epidemiology of salmonellosis in passerines in Great Britain. It might be, for example, that the greenfinch acts as an amplifier host, leading to pathogen spill-over to sympatric species during disease outbreaks.

Salmonellosis was detected more frequently in male than female greenfinches in this study. A male bias was also found in a separate study of finch mortality due to salmonellosis and colibacillosis in Scotland (Pennycott et al., 1988a). In agreement with the findings of Refsum et al. (2003), no sex bias in house sparrows with salmonellosis was detected. Potential explanations for the observed sex bias in greenfinches include a greater number of male than female greenfinches visiting

garden bird feeding stations during the winter months in Great Britain or that male greenfinches are predisposed to infectious disease due to intrinsic variation in susceptibility or differential rates of pathogen exposure (Benskin et al., 2009). Bouwman et al. (2010) recently found that healthy male house finches (*Carpodacus mexicanus*) preferentially fed near con-specific males with *Mycoplasma gallisepticum* infection under experimental conditions: no similar effect was observed for healthy female finches. These authors hypothesised that the healthy males opt for close contact rates with diseased birds of the same sex to reduce competition, however, this altered behaviour might increase their risk of pathogen exposure: a similar mechanism might operate with salmonellosis in British greenfinches. Alternatively the bright green plumage of males could render carcasses more easily found than the dull plumage of female greenfinches. Further work is required to determine which, if any, of these possible factors are responsible for the trend.

Greenfinches and house sparrows with salmonellosis were typically in thin or emaciated body condition, although statistical comparison with birds that died of trauma as a control was not possible due to insufficient sample sizes. Comparison of the body weight values for the salmonellosis cases with published ranges for apparently-healthy greenfinches (British winter male mean=28.5g, range 25.7-35.6 n=147; female mean=28.3g range 25.1-32.1 n=92, (Eley 1991)) and house sparrows (European male range 27.7-39.5g; female 24-39.5g, (Clement et al., 1993)) also indicate that the diseased birds in this study were in poor condition.

Reports exist of birds that have died of salmonellosis being in good body condition, for example house sparrows with *S.* Typhimurium DT160 in New Zealand (Alley et al., 2002), however, the majority of reports, including those from Great Britain, describe such birds to be in poor body condition (Kirkwood et al., 1995; Daoust et al., 2000). A recent study that combined faecal screening of sick birds with PME of carcasses recovered from a single site in Scotland found that poor body fat scores and low body weight were useful field indicators to identify greenfinches with *S.* Typhimurium DT56(v) infection (Grant et al., 2007).

Serotype and phage type in garden birds

In the current study, *S.* Typhimurium DT40 and DT56(v) were the most common phage types isolated and this concurs with the findings of Pennycott et al. (2006) from cases of passerine salmonellosis in Scotland between 1995 and 2003. *Salmonella* Typhimurium DT40 has been recorded previously as a common phage type associated with salmonellosis in passerines in Great Britain, Canada, Japan and Norway (Prescott et al., 1988; Pennycott et al., 1998a; Daoust et al., 2000; Refsum et al., 2002a; Tauni et al., 2000; Une et al., 2008). *Salmonella* Typhimurium DT56(v) was first recognised by the SRU in the mid 1990s and has not been reported in wild birds outside Great Britain (Hughes et al., 2008). This phage type was isolated sporadically until 2000 and 2001, when the frequency of isolation peaked, along with that of DT40, coincident with the years with the greatest number of reported salmonellosis incidents in garden birds. *Salmonella* Typhimurium DT160, which was the third most-frequently isolated phage type in this study, has been reported as a cause of sporadic mortality of small passerines in Great Britain and world-wide since the 1960s (Macdonald et al., 1969; Penfold et al., 1979; Prescott et al., 1998) and, more recently, as a cause of epizootic disease in New Zealand, principally affecting house sparrows (Alley et al., 2002).

Although the *S.* Typhimurium phage types most commonly isolated in this survey (DT40, DT56(v) and DT160) are not typically associated with livestock disease (VLA 2005; Pennycott et al., 2006), reports of DT40 and other 'wild bird' phage types in livestock, game birds and farmed poultry indicate a potential for transmission from wild birds to livestock and vice versa (Pennycott et al., 1998a, 2006). Of the remaining *S.* Typhimurium phage types isolated in this study, DT120 and DT193 have been previously isolated from livestock species, including cattle, sheep, pigs, chickens, turkey and game birds in Great Britain (VLA 2005). Information on whether the infection with these phage types was associated with clinical disease in these species is unavailable. The majority of salmonella infection incidents in ruminants and pigs, however, were detected as a result of clinical disease investigation, whilst the majority of salmonella infection incidents detected in poultry were detected as a result of active surveillance (VLA 2005).

Annual trends in garden birds and humans

Direct comparison of the number of salmonellosis incidents diagnosed between years is problematic in this study because of the opportunistic nature of reports and the inconsistent (and unknown) observer effort between years. However, both the number and the proportion of mortality incidents due to salmonellosis were much greater during the winter of 2000/2001 than in any other year. A similar peak was reported in Scotland over the same winter season (Pennycott 2001), suggesting that there was an unusually high incidence of salmonellosis across Great Britain at that time. A comparison of meteorological data (Met Office 2009) for winter seasons in Britain over the period of study failed to identify any climatic factors likely to predispose to salmonellosis during winter 2001, but these data were monthly means across the country, therefore factors such as brief periods of extreme cold or prolonged duration of snow cover would not have been detected. No contemporaneous peak was observed in the number of human *S.* Typhimurium DT40 cases.

The absolute numbers and proportions of garden bird salmonellosis incidents with each of the phage types varied across the study period, 1993–2003. DT40 was found throughout the 11-year period; however, the majority of DT160 isolates were isolated only from 1993 to 1996 while DT56(v) was mostly isolated from 2000 to 2003. Longitudinal monitoring of cattle populations in England and Germany also revealed changes in the relative importance of the *S.* Typhimurium phage types that were recovered over time (Rabsch et al., 2002). The mechanism for this phage type succession in wildlife populations is ill understood. Remarkably similar annual trends were observed in the human cases of infection due to *S.* Typhimurium DT40, DT56(v) and DT160 which supports a possible epidemiological link between infection in human and passerine populations. A positive significant correlation between the number of garden bird salmonellosis incidents and the human cases of infection with *S.* Typhimurium DT160 was found, supporting this link further. No similar significant association was found for the other most common phage types, DT56(v) and DT40, however, given the variation in observer effort for passerine disease over the study period this does not provide strong evidence to refute the postulated link.

S. Typhimurium DT40 was first recorded by the SRU in 1964. A retrospective review of SRU reports confirmed 302 human and 269 non-human isolates of DT40 from 1964 to 1973, 61% (165 reports) of the latter were from wild birds (L. Ward, *pers. comm.*). *S.* Typhimurium DT160 was first recorded by the SRU in 1966. SRU reports confirmed 556 human and 328 non-human isolates of DT40 from 1966 to 1973, 41% (134 reports) of the latter were from wild birds (L. Ward, *pers. comm.*). This historical data provides further strong evidence that wild birds are an important source of these human infections. Although *S.* Typhimurium DT56 was first recorded in 1963 by the SRU, the DT56(v) phage type was first recognised in 1995 during this period of study.

Geographical distribution in garden birds and humans

The distribution of salmonellosis incidents in garden birds across Britain was heterogenous with incidents being widespread across the English Midlands, the English/Welsh border region and southern England, but generally absent from across central England and East Anglia, even though passerine carcasses were regularly submitted from these last 2 regions. Reasons for this observed geographical variation in the occurrence of salmonellosis incidents are unclear. Comparison of the Breeding Bird Survey with these findings confirm that both the greenfinch and the house sparrow are present across England and Wales (Raven et al., 2004) and regional variations in their distributions across Britain do not match those of salmonellosis. Ring return data between 1909 and 1991 show that seasonal movement of a proportion of the greenfinch population occurs with a general trend towards the south and west between early November and late January (Main 1996), whilst the house sparrow population in the UK is sedentary in nature (Summers-Smith 1963). Whether greenfinch population movements influence the epidemiology of salmonellosis and distribution of incidents merits further investigation.

Regional variation in the distribution of passerine salmonellosis cases was recently described in the United States (Hall et al., 2008). These authors' proposed that this might relate to reduced wildlife habitat and increased human density in affected regions

leading to increased congregation at feeding stations. This explanation does not apply to the situation in Britain where some of the areas with high salmonellosis (e.g. English/Welsh border region) are typically rural with less-dense human habitations. Other land use practices vary across Britain, with, for example, a greater concentration of arable farming in East Anglia (where salmonellosis incidence in garden birds appeared to be low) and a greater concentration of livestock farming in southern and western England (where salmonellosis incidence appeared to be high). In order to test how meaningful such factors might be, however, a systematic approach with standardised observer effort is required.

A positive spatial relationship was found between the human infections and the garden bird mortality incidents for *S.* Typhimurium DT40, DT56(v) and DT160 which provides evidence of a possible epidemiological link between these 2 groups. It is possible that both birds and people become infected from the same independent source, which could result in the similar annual trends and geographical distributions observed; however, infection with these *S.* Typhimurium phage types is infrequently seen in livestock, companion animals and other wildlife species making this explanation unlikely (Euden 1990; Pennycott et al., 2006; Immerseel et al., 2004). The most plausible explanation for these findings it that garden bird populations are the major source of human infection with these phage types.

Seasonal trends in garden birds

A strong seasonal effect, with a peak incidence of passerine salmonellosis in winter/early spring, was found in each year of this study and similar findings have been described for incidents previously in Europe (Faddoul et al., 1966; Refsum et al., 2002a; Pennycott et al., 2002) and North America (Hall et al., 2008), although year-round cases have been observed in some studies (Daoust et al., 2000). Seasonal variation in the GBW species reporting rates (percentage of sites with positive sightings) showed mean quarterly values for the greenfinch of 68% in October-December and 79% in April-June; 76% and 82% in the same months, respectively, for the house sparrow (Toms 2003). This confirms that these species are present in a large proportion of garden habitats

throughout the calendar year in Britain so the apparent disease seasonality does not relate to temporal variation in habitat utilisation. Some authors (e.g. Refsum et al., 2003) have suggested that the apparent winter seasonality may be an artifact since observer effort is likely to be greatest during the winter season when, traditionally, greater focus is placed on wild bird provisioning. Nevertheless, there is an increasing trend toward year-round feeding of garden birds in Great Britain (Routh et al., 1995). In the current study, the large numbers of bird carcasses submitted during the summer and autumn months from garden habitats with no evidence of salmonellosis indicate that salmonellosis is indeed a seasonal disease of garden birds.

Possible explanations for winter seasonality include resource limitation and competition (e.g. food or other factors), inclement weather (e.g. cold, wet) or other physiological stress, leading to secondary immunosuppression (Wilson et al., 1967). Adequate food supplies within the local environment were documented during a Canadian salmonellosis outbreak, consequently starvation was not thought to be a contributing factor in that case (Daoust et al., 2000). Also, outbreaks in the current study typically involved sites of provisioning, suggesting that starvation was not a predisposing factor to salmonellosis.

Seasonal trends in humans

The garden bird-associated *S.* Typhimurium phage types were isolated from humans throughout the calendar year: the seasonal pattern differed between the 3 phage types. This contrasts with the epidemiology of salmonellosis in garden birds where morbidity and mortality consistently peaks during the winter season. *S.* Typhimurium DT56(v) human cases of infection peaked in the winter, as did DT40 cases although the latter were spread more evenly across the seasons: DT160 human cases peaked in the summer. The explanation for this variation among the seasonality of human and garden bird infection with these *S.* Typhimurium phage types is not readily apparent. This winter seasonality for DT40 and DT56(v) human cases, the phage types most frequently isolated from passerines, contrasts with the typical late summer peak of *S.* Typhimurium

infection (all phage types) in humans: the HPA reported 36-40% of all human cases of *S.* Typhimurium infection occurred from July to September, 2001 to 2003 (HPA 2010).

Kapperud et al. (1998) found a close temporal relationship between garden bird *S.* Typhimurium O:4-12 variant infection in humans and wild birds in Norway, both peaking between January and April. Year-round feeding of garden birds is commonly practiced in Great Britain in contrast to Norway where winter feeding predominates (Refsum et al., 2003); this could influence the seasonality of human exposure. Screening of wild bird faeces from a bird table at a feeding site with a history of salmonellosis in Scotland identified a peak of positive samples in the winter months; however, *S.* Typhimurium DT56(v) was recovered throughout the calendar year with a second peak in August - September. This indicates that continued excretion or persistence of infection may occur at sites for some *S.* Typhimurium phage types (Pennycott et al., 2002).

Humans may become infected with garden bird *S.* Typhimurium phage types via direct or indirect routes of transmission. Handling of sick and dead garden birds with salmonellosis has been identified as a risk factor for human infection (Kapperud et al. 1998; Thornley et al., 2003). Handling clinically healthy birds excreting *Salmonella* spp. also has the potential to result in infection, with licensed bird ringers and ornithologists being a demographic group at risk of exposure (Abulreesh et al., 2007). Whilst little information is available on the rates of asymptomatic carriage of these salmonella strains in apparently healthy garden birds in Great Britain, available data screening asymptomatic passerine populations in Europe (Palmgren et al., 1997, Hernandez et al., 2003; Refsum et al., 2003) indicate that the risk of salmonella infection associated with handling healthy passerines is likely to be considerably lower than during a salmonellosis outbreak.

The winter peak observed in human cases for *S.* Typhimurium DT40 and DT56(v) is consistent with direct transmission of infection to humans during the passerine salmonellosis season. However, *S.* Typhimurium DT160 peaked during the summer, and

cases of infection with each of the phage types were observed throughout the calendar year, consequently direct transmission may not represent the most important, or sole, route of transmission between wild birds and humans.

Human infection could also result through indirect routes of exposure, for example contact with bridge species (livestock, companion animal such as cats and horses and wildlife). Healthy house cats are generally considered a low risk for salmonella excretion; Immerseel et al. (2004) screened rectal swabs from 278 healthy house cats and recovered a *Salmonella* sp. isolate from a single cat only (*Salmonella* enteriditis phage type 21). Consequently cats are unlikely to pose a significant risk of infection to humans unless they are acting as bridge species following infection through predation of infected birds. Risk activities for human infection could include handling of cat litter trays, particularly during a period of concomitant salmonellosis in garden birds and nursing of sick pets. In Sweden, 8% (5/64) of individuals who owned cats with salmonellosis experienced gastrointestinal tract problems themselves; faecal samples were submitted from 4 of these individuals and none tested culture positive for *Salmonella* spp. (Tauni et al., 2000). Four people presented with enteric disease due to the garden bird *S.* Typhimurium phage types during an outbreak of salmonellosis in cats and garden birds in the first half of 1999 in Sweden of which 2 had a history of sick cats in their household and the other 2 people fed wild birds (Tauni et al., 2000). Contact with sick cats is considered to be a more common source of infection for humans than direct contact with wild birds by some authors (Tizard 2004). Human infections that result from contact with sick cats might be predicted to peak during the winter months when passerine salmonellosis, and therefore infected prey, predominates: since winter seasonality was observed for 2 of the phage types in human cases, indirect transmission from companion animals may represent a route of infection, particularly for these *S.* Typhimurium DT40 and DT56(v), although, its relative importance is unknown.

Indirect transmission of infection to humans also might occur via contact with environmental sources (e.g. garden soil) contaminated with wild bird faeces. In a Norwegian study, 2 sites with salmonellosis incidents in garden birds on multiple years

recovered *Salmonella* spp. from waste food and the soil indicating that infection has the potential for environmental persistence at sites between years (Refsum et al., 2003). Numerous factors influence persistence of *Salmonella* spp. in the environment including temperature, moisture, soil type, presence of manure contamination, UV exposure, and season of contamination. Experimental studies provide evidence for long-term environmental persistence, for example *S.* Typhimurium was found to persist 96 days at 8 °C (Tamási 1981), 180 days in manure-contaminated soil during simulated summer-winter exposure (Holley et al., 2006), and 280 days in urban garden soil (Friend 1999a). The ability of *Salmonella* spp. to persist for long yet variable periods in the environment could provide an explanation for the lack of consistent seasonality observed in the human infections, and the summer peak observed in *S.* Typhimurium DT160 isolates. Indeed the seasonality of the activity that leads to human exposure may be an important determinant of the timing of human cases, for example gardening and outdoor recreation activities might increase the risk of exposure in the summer.

In this study the date of submission of the isolate was available, rather than the time of first clinical case presentation. A time lag exists between the time of exposure and the culture diagnosis, including the period of incubation and clinical disease that prompted presentation for medical investigation, therefore the actual exposure event that resulted in the infection will pre-date the time of isolate submission and this should be borne in mind when discussing seasonal risk activities that may indirectly expose humans to infection.

The variable seasonality in the occurrence of human cases of infection with garden bird-associated *S.* Typhimurium phage types indicates that a combination of direct and indirect routes of exposure are likely. Unfortunately detailed case history information was not available for the patients in this study; consequently risk factor analyses which might further inform routes of transmission could not be performed. Thornley et al. (2003) investigated an outbreak of *S.* Typhimurium DT160 infection in humans that occurred when this strain of the bacterium first emerged as a cause of garden bird mortality in New Zealand. The risk factors for human infection that were identified in

this study included contact with dead wild birds, contact with other people with gastrointestinal disease, consumption of fast food and drinking of untreated roof-collected rain water (Thornley et al., 2003).

In a prospective case control study of human infections with *S.* Typhimurium O:4-12 in Norway, patients were questioned about the two-week period preceding the onset of their clinical signs. Detailed questionnaires explored multiple potential risk factors and concluded that drinking untreated water, direct contact with wild birds or their droppings and ingestion of snow, sand or soil were related to an increased risk of infection. Of the 10 respondents who reported a history of direct contact with wild birds or their droppings, 6 had cleaned a bird feeder or removed bird faeces and 4 had touched a dead bird or nursed a sick bird. No increase in risk was observed for people who fed garden birds, or for other family members who shared a household where bird feeding was practiced. Also, there was no increased risk for people in contact with wild, captive or domestic animals (Kapperud et al., 1998).

Age composition of human infection

Infants were the modal age group confirmed with human infection for each of the garden bird-associated *S.* Typhimurium phage types in this study which mirrors the general trend reported by the HPA for all *S.* Typhimurium phage types. Between 1993 and 2003, the HPA identified salmonella infection in humans (excluding *S.* Typhi and *S.* Paratyphi) from faecal or lower gastrointestinal tract isolates in 285.1 cases per 100,000 individuals for infants (0-5 years old), 71.9 cases per 100,000 individuals for children (5-14 years old), 74.9 cases per 100,000 individuals for adult (15-64 years old) and 48.4 cases per 100,000 individuals for geriatric (>65 years old), from England and Wales.

Limited information is available regarding the demography of human infection with garden bird *S.* Typhimurium strains. In Norway, 43% of the 153 human cases of infection with that outbreak's garden bird strain of *S.* Typhimurium were less than 5 years old (Kapperud et al., 1998). Similarly, the Scottish Salmonella Reference Unit between 2001 and 2007 found that 38% of the 47 *S.* Typhimurium DT40 isolates and

52% of the 29 DT56(v) isolates in humans were from children less than 5 years old (Philbey et al., 2009). In New Zealand, the median age of patients with *S.* Typhimurium DT160 was 8 years (Thornley et al., 2003). In this study, 44% of human infections were in the infant age category and no significant difference was found in the age groups affected for each of the 3 *S.* Typhimurium phage types.

Evidence suggests that infants, the immunocompromised and elderly people are more susceptible than adults to infection following challenge with lower numbers of a *Salmonella* sp. (Baird-Parker 1991). Infants are more likely to be immunologically näive to salmonella infection, as compared with adults where immunity may have developed, contributing to this age trend in cases (Smith et al., 1964). Infants may be more likely to be presented for clinical investigation of gastroenteritis compared with older patients where symptomatic management may be elected. The average number of GP consultations per year was 5.5 for infants (0-4 years) between 1998 and 2002 as compared with 2.5 per year for children (5-15 years) for the same period (Rickards et al., 2004). In addition, infants and children are likely to have poorer levels of personal hygiene which, coupled with year-round play in outdoor environments, may make them more likely to be exposed to environmental *Salmonella* spp. bacteria than older demographic groups.

Significance of human infection

The patterns of human infection with garden bird-associated *S.* Typhimurium phage types in England and Wales mirrored those found in garden birds temporally and spatially for DT40, DT56(v) and DT160, providing evidence of a potential epidemiological link. However these findings must be viewed in context. Between 1993 and 2004, the HPA survey of salmonella infection in humans in England and Wales (excluding *S.* Typhi and *S.* Paratyphi) from faecal or lower gastrointestinal tract isolates identified 246,009 *Salmonella* spp. isolates (40,193 *S.* Typhimurium) therefore the garden bird *S.* Typhimurium isolates DT40, DT56(v) and DT160 account for only 0.13% of all the *Salmonella* spp. isolates and 0.78% of the *S.* Typhimurium isolates (HPA 2005).

However, the number of cases of salmonellosis in humans is likely to be under-reported. People with mild cases of gastroenteritis may not present to the medical community for further investigation and, if they do, samples might not be submitted for bacteriological analysis.

Antimicrobial susceptibility studies of *S.* Typhimurium isolates from garden birds in the north of England show minimal antibiotic resistance indicating that they do not represent an important source of resistant infection to the human population (Hughes et al., 2008). These authors' also determined that these isolates lacked the translocated effector protein (sopE) gene which has been frequently associated with some epidemic strains of *S.* Typhimurium in humans and livestock indicating that wild birds may not pose a high zoonotic risk (Hopkins et al., 2004; Hughes et al., 2008).

Studies of human infection with *S.* Typhimurium garden bird phage types have focused on countries with current or recent epidemics of passerine salmonellosis rather than the more endemic pattern of localised disease incidents observed in Great Britain. Heir et al. (2002) found that 32% of sporadic domestically acquired human infections in Norway between 1996 and 1999 were with the 0:4,12 strain of *S.* Typhimurium infection which had an identical PFGE profile to passerine isolates and concluded that wild birds represent a continued and important source of infection for humans. During an outbreak of *S.* Typhimurium DT160 infection in passerines in New Zealand, 180 human cases of infection occurred in 2000 which accounted for 9% of the total cases for that year (Thornley et al., 2003).

In a Norwegian study, 46% (19/41) of patients were admitted to hospital for treatment of salmonellosis for a mean of 5.2 days; whilst the overall number of human cases accounted for a small proportion of salmonellosis in humans over their study period this illustrates that the result of infection can be significant (Kapperud et al., 1998). Three of the 4 cases of human infection with S. Typhimurium DT40 infection reported following contact with sick cats or wild birds were hospitalized (Tauni et al., 2000). In New

Zealand, patients with DT160 had a median duration of illness of 7 days (range 1-44 days) of which 15% (17/170) were hospitalized for treatment (Thornley et al., 2003). Patients with severe gastroenteritis due to DT160 infection in New Zealand in 2000 had a higher rate of hospitalization than with other *Salmonella* spp. at that time (Connolly et al., 2006). No information was available on the clinical history or course for patients in this study. The severity and outcomes of human infection in Great Britain should be appraised in the future.

The importance of garden birds as a potential reservoir of *Salmonella* Typhimurium and source of zoonotic infection must remain in perspective and there is no evidence for a growing cause for concern. Sensible routine hygiene precautions are recommended to reduce the risk of exposure. Members of the public who feed garden birds should be aware that wild birds can carry zoonotic pathogens such as salmonella. Information on common causes of garden bird disease (Pennycott et al., 1998b), the risk of secondary infection in companion animals and measures for diagnosis and control where disease outbreaks occur (Tizard 2004) is available along with best practice guidance for feeding of garden birds (GBH*i* 2006) and for licensed bird ringers (Abulreesh et al., 2007).

Molecular epidemiological studies using PFGE, PCR virulotyping or multilocus sequence typing on isolates of *S.* Typhimurium from garden birds and humans are required to investigate further whether the same strains of *S.* Typhimurium infect both groups. This work may help to elucidate the role that passerines might play as a source of zoonotic infection in Great Britain.

CHAPTER 3: COMPARING OPPORTUNISTIC AND SYSTEMATIC APPROACHES FOR SURVEILLANCE OF THE CAUSES OF MORTALITY IN BRITISH GARDEN BIRDS, 2005 TO 2008

3.1 INTRODUCTION

Citizen science methodologies offer a promising solution to achieve financially viable surveillance of the causes of wildlife mortality events across large geographical regions. Such schemes have been used for the monitoring of specific pathogens with clear external clinical signs (e.g. Dhondt et al., 1998). Alternatively, cause of death categories have been assigned based on information from the reporter on the circumstances of finding (e.g. cat kill, window collision): however, no confirmatory pathological investigations were performed in these studies (e.g. Brittingham et al., 1986). A large scale citizen science project combining public reporting and post mortem examination has not yet been performed.

Schemes to monitor wildlife mortality include opportunistic or systematic reporting networks. Opportunistic (i.e. *ad hoc*) reporting of wildlife mortality incidents by the general public has the advantage of low cost, and the potential for national coverage, in addition to engaging public interest and increasing education in wildlife population and ecosystem health. However, the inconsistent temporal and spatial effort in the monitoring that can be achieved limits interpretation of these findings (Eidson et al., 2001). Whilst it is possible to document the presence of infectious disease at sites where investigations have been performed, it is not possible to conclude with any confidence on the absence of disease with surveillance based on opportunistic submissions. Reporting of mortality incidents may be biased to those affecting species with high visibility, for example due to plumage colouration, or charismatic species. Media attention or public education campaigns may artefactually elevate reporting rates, complicating comparison of findings between years or seasons (Mörner et al., 2002). These limitations underline the importance of systematic schemes with consistent

monitoring effort in time and space which enable comment on the presence and probable absence of mortality events.

In 2005, the Universities Federation for Animal Welfare brought together a Working Group on Feeding garden birds including representatives from ornithological non-governmental organizations (British Trust for Ornithology (BTO) and the Royal Society or the Protection of Birds (RSPB)), disease investigation centres with an established interest in wild bird disease research (Institute of Zoology, Scottish Agricultural College, University of Liverpool, Wildlife Veterinary Investigation Centre) and representatives of the garden bird food industry. This 3-year research project (called the Garden Bird Health *initiative* (GBH*i*)) was established to investigate causes of garden bird mortality across GreatBritain using a combination of opportunistic and systematic methodologies in a citizen science project.

This chapter describes the novel surveillance approach used in this study for monitoring causes of mortality in British garden birds combining both opportunistic and systematic methodologies, in conjunction with pathological investigations performed at a regional network of disease investigation centres.

The relative benefits and costs of both the opportunistic and systematic schemes are compared and potential biases in the findings and cases submitted for post mortem examination are examined. The ability of the systematic participant network to identify cause of death categories is evaluated. Recommendations are drawn for schemes used to monitor causes of wildlife mortality in the future.

3.2 MATERIALS AND METHODS

Opportunistic reports of garden bird morbidity and mortality were solicited from the general public through the RSPB Wildlife Enquiries Unit, and other wildlife and animal welfare non-governmental organizations, and reported to the GBH*i* via a dedicated telephone reporting line between 1st April 2005 and 31st March 2008. A full history was

recorded, supported by a questionnaire (Appendix 1) on garden bird feeding (e.g. number and type of feeders, type and volume of feed, hygiene practise) at the site and details of the species and number of birds visiting the feeding station. Members of the public with opportunistic reports of mortality were directed to a regional disease investigation centre dependent on the history and availability of carcasses in a suitable condition for post mortem examination (PME).

Systematic surveillance was achieved through a link with the established BTO Garden BirdWatch scheme (GBW) of approximately 15,000 participants throughout Great Britain (Cannon et al., 2005). A stratified, random sample of GBW participants, with even geographical coverage across Great Britain and a range of garden bird feeding practise, were approached to supply information on observations of sick or dead birds in their gardens on a weekly basis between 1st October 2005 and 31st March 2008: 751 participants were recruited and active at the outset of the study period.

Participants were asked to search their gardens with consistent effort across recording weeks and to record presence or absence of garden bird morbidity and mortality. In addition, they provided details of the type and volume of feed supplied to the garden birds, the number and design of feeders in use and the hygiene measures employed at the feeding station (Appendix 2). For the first-year, hand-written reporting forms were used where participants recorded the weight of foodstuffs provided on a weekly basis (Appendix 3). For the second and third year of study, tick-box forms were used where the participant classified the amount of each foodstuff provided as 'small', 'medium' or 'large' according to the range of actual values reported for all participants in the first year (Appendix 4). Participants were requested to record all garden bird mortality and provide information on the cause of death (COD) they suspected to be responsible in each case based on the circumstances of finding. The BTO GBW co-ordinator independently reviewed this information and allocated a COD for each case based on the following categories: 'Infectious disease', 'Predation', 'Other trauma', 'Other' and 'Not established'. 'Other trauma' included circumstances such as window collision and road

traffic accident injuries. 'Other' included miscellaneous circumstances such as drowning or euthanasia.

When a garden bird carcass in appropriate condition for PME was available the participants were requested to contact their nominated regional disease investigation centre. It was emphasized that examination of birds from all suspected COD categories (i.e. including victims of cat predation and window collision) was important to the study.

PMEs were performed on carcasses submitted from a subset of reported mortality incidents using standardised examination and microbiology protocols at each centre (Chapter 4). Appropriate guidelines for postal submission of pathological specimens including relevant health and safety advice were provided (Appendix 2). A range of techniques including gross pathology, microbiology, parasitology, histopathology, electron microscopy, and polymerase chain reaction were employed (Chapter 4). A definitive COD was assigned based on the PME findings using the same categories (see above).

The findings from the opportunistic and systematic monitoring schemes were compared including the number of birds, species complement, seasonality and COD categories of those examined post mortem. The seasons were classified as winter (Dec – Feb), spring (March – May), summer (June –Aug) and autumn (Sept – Nov). The geographical distribution of sites from which birds were submitted was evaluated for both schemes to assess their efficacy in terms of national surveillance.

For the systematic scheme, potential reporting biases in the species, season and COD categories were established by comparing the total dead birds reported with the total birds submitted for PME. The suspected COD reported by the participant on submission of each case was compared with the actual COD confirmed on PME to evaluate the confidence that can be placed in the conclusions of the systematic participants, in the absence of pathological investigations, and to estimate the overall importance of

infectious disease as a significant contributory factor to garden bird mortality across the study period.

Non-parametric analyses including Pearson chi-square test, Fishers exact test and the binomial test of proportions, as appropriate, were performed using SPSS 17.0 for windows (SPSS Inc., Chicago, U.S.A.) and R –CRAN (http://www.R-project.org). Spatial data were presented using ArcView 3.0 geographical information system (GIS) software (Environmental Systems Research Institute GIS and Mapping Software, California, U.S.A.).

3.3 RESULTS

Comparison of the opportunistic and systematic schemes

Between 1st April 2005 and 31st March 2008, 1,559 PMEs were performed across all the disease investigation centres (495 carcasses in Year 1 (1st April 2005 – 31st March 2006), 626 in Year 2 (1st April 2006 – 31st March 2007) and 438 in Year 3 (1st April 2007 – 31st March 2008)). These comprised, from the opportunistic surveillance scheme, 929 carcasses (of 40 species) from 626 sites, and, from the systematic surveillance scheme, 630 carcasses (of 39 species) from 251 sites. A significantly greater proportion of cases were submitted through the opportunistic than systematic scheme across the study period (X^2=55.443, df=1, P<0.0001) and the frequency of PME for each of the schemes differed significantly by year (X^2=15.758, df=2, P<0.0001). The percentage of all PME submissions that were from the systematic scheme varied between 46% (288/626) of birds in Year 2 and 34% (19/438) of birds in Year 3 (Figure 3.1).

Figure 3.1: Percentage of all PME submissions in the opportunistic and systematic schemes by year of the study period: Year 1 (1st April 2005 – 31st March 2006), Year 2 (1st April 2006 – 31st March 2007), Year 3 (1st April 2007 – 31st March 2008).

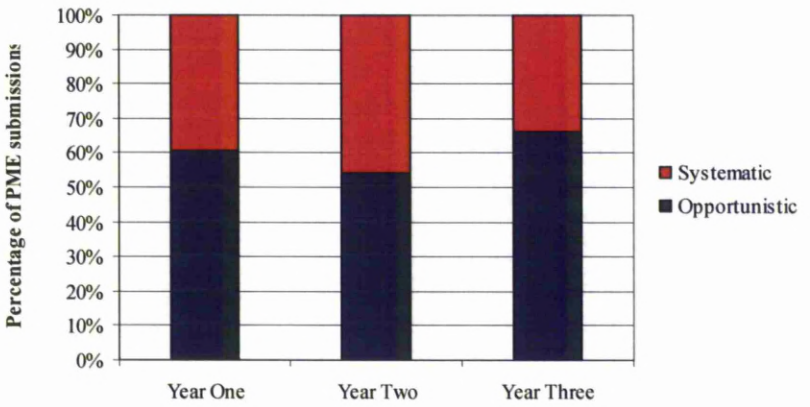
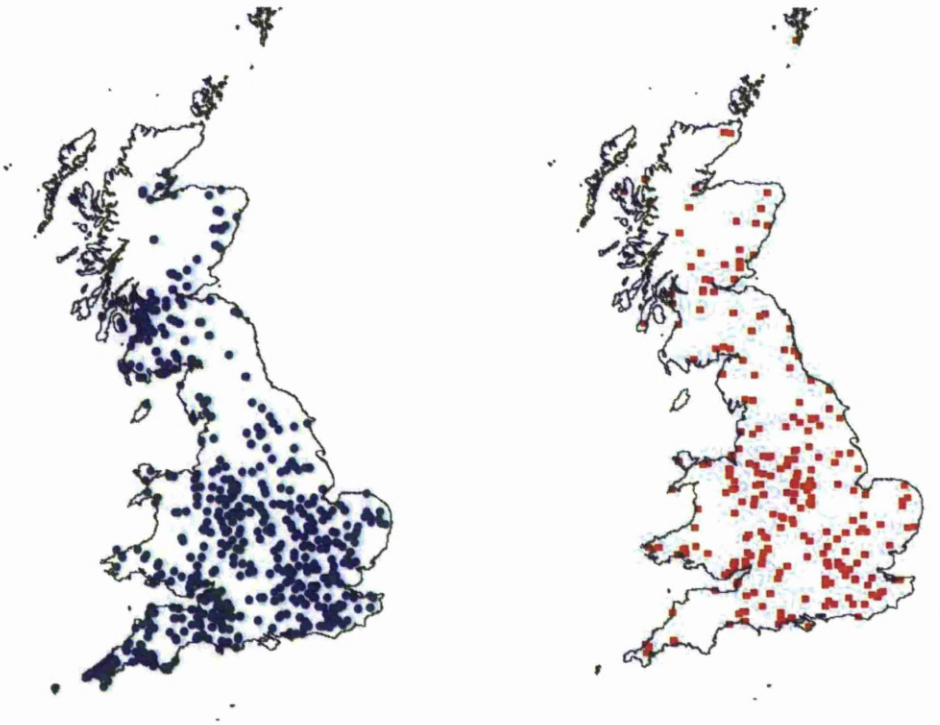


Figure 3.2: Geographical distribution of opportunistic (a) and systematic (b) PME submissions (1st April 2005 – 31st March 2008).

Opportunistic (a) Blue circles represent sites from which birds were submitted for PME

Systematic (b)Red squares represent sites from which birds were submitted for PME



Excellent geographical coverage was achieved through both the opportunistic and systematic schemes with birds submitted for PME from across Great Britain (Figure 3.2).

Greenfinches (*Carduelis chloris* n=539) and chaffinches (*Fringilla coelebs* n=276) were the most frequently examined species in rank order. Comparison of the species population distribution from the 2003 Breeding Bird Survey data (Raven et al., 2004) with PME submissions for these species confirmed that comprehensive national coverage was achieved in both surveillance schemes (Figure 3.3).

Figure 3.3: Distribution of PME submissions (1st April 2005 – 31st March 2008)

(a) Greenfinch

Opportunistic scheme PME submissions

Systematic scheme PME submissions

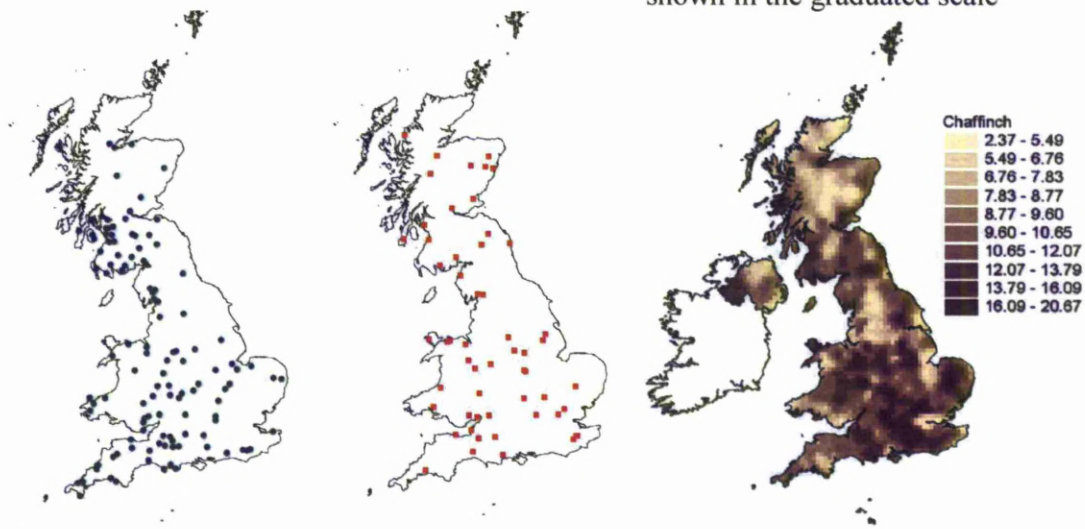
Breeding Bird Survey 2003 Relative abundance estimates shown in the graduated scale

(b) Chaffinch

Opportunistic scheme PME submissions

Systematic scheme PME submissions

Breeding Bird Survey 2003 Relative abundance estimates shown in the graduated scale



Comparison of the number of PME submissions for each avian family (with total submissions > 5 birds) identified a significant difference between the opportunistic and systematic schemes (X^2=56.914, df=12, P<0.0001). The binomial test of proportions found no significant difference between the opportunistic and systematic schemes for the Accipitridae, Aegithalidae, Columbidae, Corvidae, Emberizidae, Passeridae, Paridae, Picidae, Prunellidae, Sturnidae, and Sylviidae (Table 3.1). Fringillidae species were significantly more frequently submitted through the opportunistic than systematic scheme (X^2=20.457, df=1, P<0.0001); the converse was true for the Turdidae species (X^2=21.623, df =1, P<0.0001).

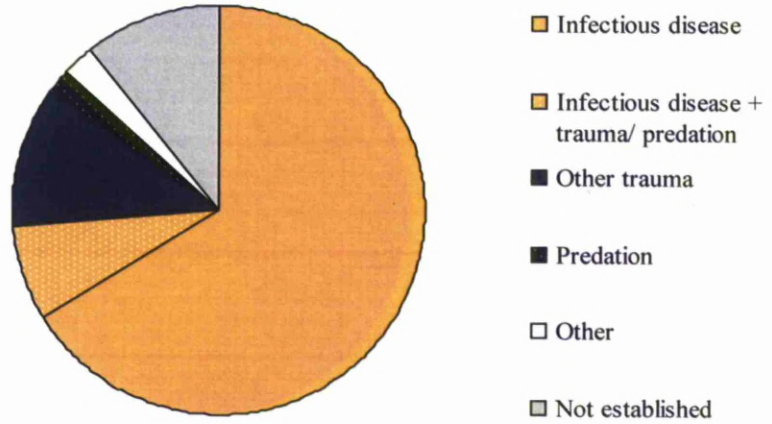
Table 3.1: Comparison of the avian family breakdown of PME through the opportunistic and systematic schemes (1st April 2005 – 31st March 2008). Total number (and overall percentage) of submissions shown for each scheme.
*** denotes significant difference between the schemes at the P=0.001 level
n.s. denotes no significant difference between the schemes

Bird Order/ Family	Opportunistic PME submissions (%)	Systematic PME submissions (%)	Binomial test of proportions	Total PME submissions (%)
Accipitriformes/ Accipitridae	7 (0.8%)	2 (0.3%)	n.s.	9 (0.5%)
Columbiformes/ Columbidae	51 (5.5%)	38 (6.0%)	n.s.	89(5.7%)
Passeriformes/ Corvidae	5 (0.5%)	5 (0.8%)	n.s.	10 (0.6%)
Passeriformes/ Emberizidae	9 (1.0%)	3 9 (0.5%)	n.s.	48 (3.1%)
Passeriformes/ Fringillidae	626 (67.4%)	356 (56.5%)	***	982 (63%)
Passeriformes/ Passeridae	80 (8.6%)	39 (6.2%)	n.s.	119 (7.6%)
Passeriformes/ Paridae	27 (2.9%)	35 (5.6%)	n.s.	62 (4.0%)
Passeriformes/ Prunellidae	18 (1.9%)	24 (3.8%)	n.s.	42 (2.6%)
Passeriformes/ Sturnidae	18 (1.9%)	13 (2.0%)	n.s.	31 (2.0%)
Passeriformes/ Syliviidae	3 (0.3%)	6 (1.0%)	n.s.	9 (0.5%)
Passeriformes/ Turdidae	55 (5.9%)	82 (13.0%)	***	137 (8.8%)
Piciformes/ Picidae	3 (0.3%)	9 (1.4%)	n.s.	12 (0.7%)

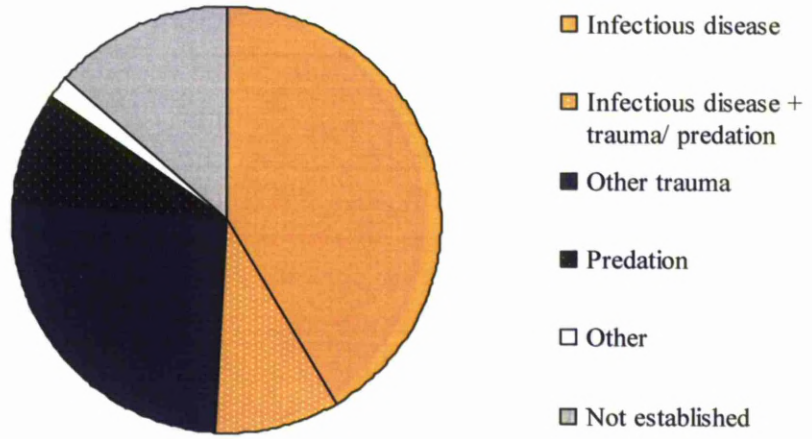
There was a significant difference in the frequency of submissions between the opportunistic and systematic schemes across the COD categories (X^2=126.480, df=5, P<0.0001) (Figure 3.4). The binomial test of proportions found that a significantly greater proportion of opportunistic than systematic submissions died solely as a result of 'Infectious disease' (X^2=91.615, df=1, P<0.0001); or of significant 'Infectious disease' in combination with either 'Predation' or 'Other trauma' (X^2=84.969, df =1, P<0.0001). The converse was true for 'Predation' (X^2=34.825, df =1, P<0.0001) and 'Other trauma' (X^2=55.046, df =1, P<0.0001) COD categories.

Figure 3.4: COD categories for (a) opportunistic (n= 929 cases) and (b) systematic (n= 630 cases) PME submissions (1st April 2005 – 31st March 2008).

(a)



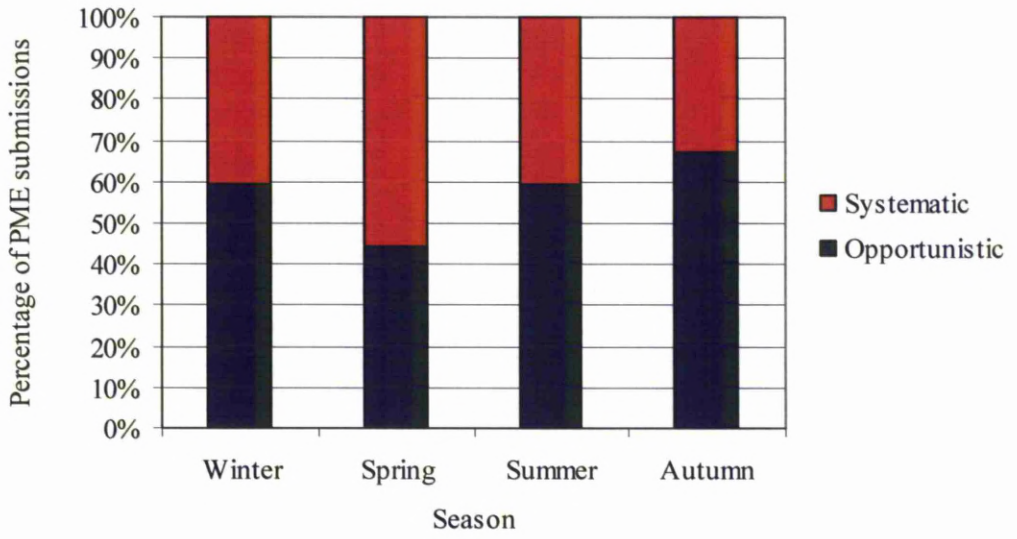
(b)



The percentage of cases where the COD category was 'Not established' was similar (11-14% of cases) for PME examinations across project years, and for both opportunistic and systematic scheme submissions. Similarly, the percentage of cases in the 'Other' COD category was 2% for PMEs across study years, and for both opportunistic and systematic scheme submissions. The binomial test of proportions found no significant difference between the opportunistic and systematic schemes for 'Not established' and 'Other' COD categories.

The proportion of PME submissions varied between the opportunistic and systematic schemes by season (Figure 3.5). A significantly greater proportion of birds were submitted in spring from the systematic scheme (X^2=25.683, df =1, P<0.0001): conversely the greater proportion was from the opportunistic scheme in autumn (X^2=15.544, df =1, P<0.0001).

Figure 3.5: Seasonal breakdown of opportunistic and systematic PME submissions (1st April 2005 – 31st March 2008). Winter (Dec – Feb), spring (March – May), summer (June –Aug), autumn (Sept – Nov).

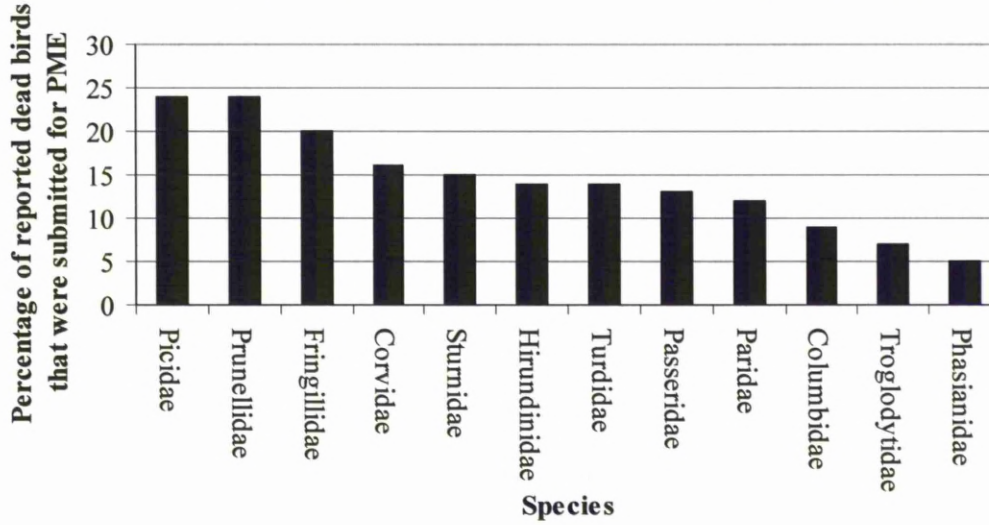


Overall bird mortality reported by systematic surveillance and evaluation of submission bias

The systematic scheme participants reported a total of 4,021 dead birds of 61 species from 31 families from 1st September 2005 – 31st March 2008. Bird families within this dataset, not represented in the PME submissions for either the opportunistic or systematic schemes, included Alcidae (n=1), Anatidae (n=5), Certhidae (n=1), Laridae (n=1), Muscicapidae (n=3), Rallidae (n=2) and Scolopacidae (n=2).

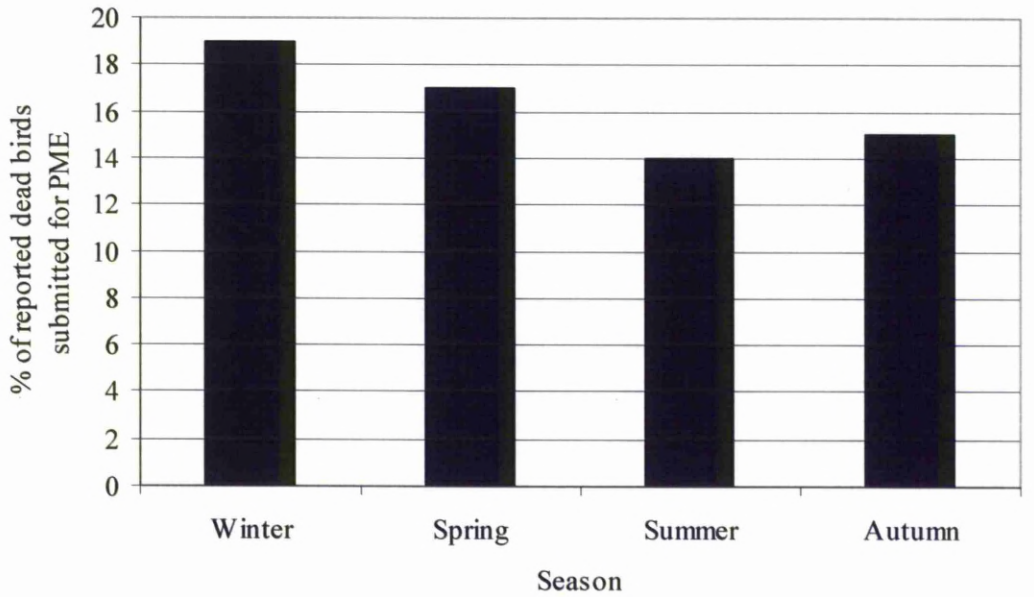
The percentage of the total number of dead birds reported from each bird family that were actually submitted for PME varied from the least frequently submitted, for example 5% of Phasianidae (1/21 birds), 7% of Troglodytidae (2/27 birds) and 9% of Columbidae (38/441 birds); to the most frequently submitted, for example 24% of Picidae (8/24 birds), 24% of Prunellidae (25/106 birds) and 20% of Fringillidae (357/1795 birds) (Figure 3.6). The frequency of reported birds that were submitted for PME varied significantly among avian families (X^2=56.34, df=11, P<0.0001) indicating that submission bias occurs according to species: avian families where 20 or more dead birds were reported across the study period were included in this analysis.

Figure 3.6: Percentage of the total number of dead birds from each family that were reported that were actually submitted for PME by the systematic scheme participants (1st October 2005 – 31st March 2008). Families with >20 dead birds reported are shown.



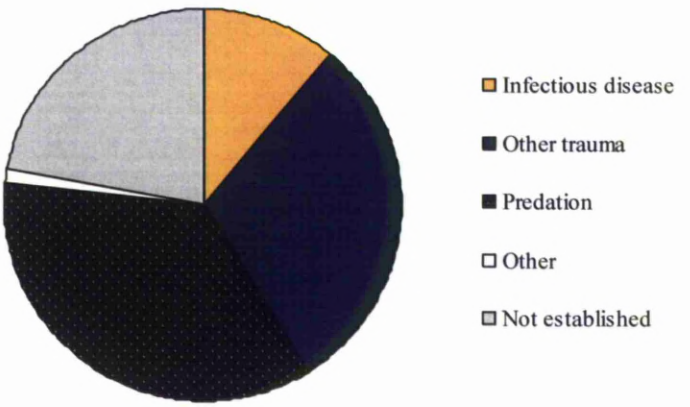
The frequency of birds found dead that were actually submitted for PME varied significantly among seasons (X^2=11.815, df=3, P=0.008), ranging from 14% in summer (178/1279 birds) to 19% in winter (158/836 birds) (Figure 3.7).

Figure 3.7: Percentage of the total number of dead birds from each season that were reported that were actually submitted for PME by the systematic scheme participants (1st October 2005 – 31st March 2008).
Winter (Dec – Feb), spring (March – May), summer (June –Aug), autumn (Sept – Nov).



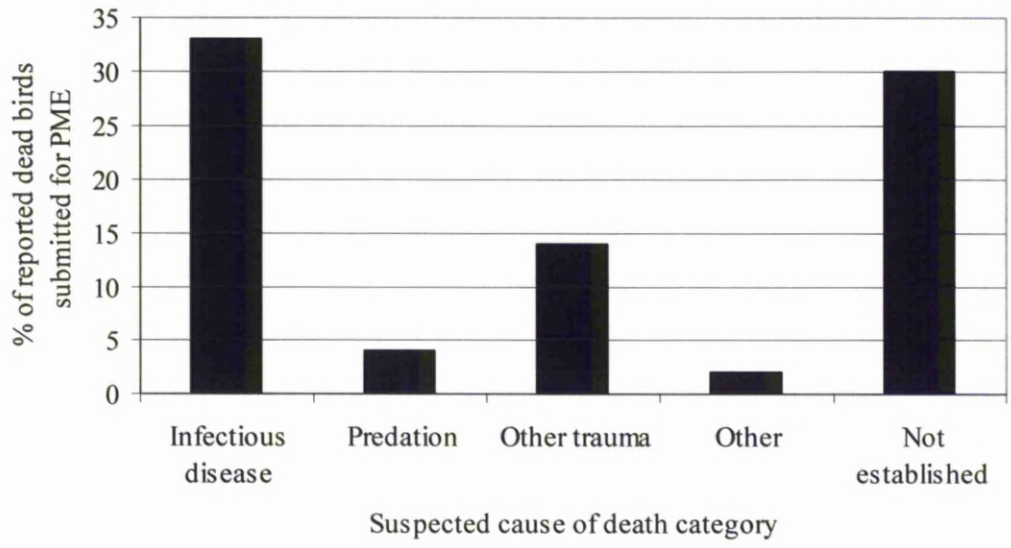
The breakdown of COD death category suspected by systematic scheme participants in all birds found dead over the study period is shown in Figure 3.8. Physical categories accounted for the majority of dead birds, with 'Predation' and 'Other trauma' (combined) accounting for 66% (2639/4021) of birds.

Figure 3.8: Breakdown of suspected COD category for all dead birds (n=4021 cases) reported by the systematic scheme participants (1st October 2005 – 31st March 2008).



The percentage of dead birds for each of the suspected COD categories, based on the observations of the systematic scheme participants, which were submitted for PME is shown in Figure 3.9. Highly significant variation was seen between categories (X^2=404.405, df=3, P<0.0001) indicating strong submission bias according to COD death category: only 4% of suspected 'Predation' cases (51/1429 birds) were submitted for PME as compared with 33% of suspected 'Infectious disease' cases (145/442 birds).

Figure 3.9: Percentage of dead birds from each suspected COD category that were reported that were actually submitted for PME by systematic scheme participants (1st October 2005 – 31st March 2008).



Evaluation of the ability of the systematic network to assign COD category

The suspected cause COD for all birds submitted through the GBW network is compared with the actual COD confirmed at PME in Table 3.2.

Table 3.2: Summary of suspected against confirmed COD (%) based on PME findings. Cells where the suspected COD and confirmed COD are in agreement are highlighted in yellow.

	Confirmed COD on PME						
Suspected COD by systematic scheme participants	Infectious Disease	Infectious Disease + Other trauma / predation	Other	Other trauma	Predation	Not established	Total
Infectious disease	112 (77%)	14 (10%)	4 (3%)	3 (2%)	1 (1%)	11 (8%)	145 (100%)
Other trauma	9 (5%)	11 (6%)	0 (0%)	113 (66%)	9 (5%)	28 (16%)	170 (100%)
Predation	7 (13%)	14 (27%)	0 (0%)	7 (13%)	23 (44%)	1 (2%)	52 (100%)
Not established	125 (48%)	28 (11%)	7 (3%)	38 (14%)	21 (8%)	44 (17%)	263 (100%)
Total	253	67	11	161	54	84	

For the cases where the suspected COD by the systematic scheme participants was 'Infectious disease', 'Infectious disease' was confirmed as a sole or contributory factor to the COD on PME in 87% (126/145) of birds. For the cases where suspected COD by the systematic scheme participants was 'Not established', 'Infectious disease' was confirmed as a sole or contributory factor to the COD on PME in 58% (153/263) of birds. For the cases where the suspected COD by the systematic scheme participants was 'Predation', 'Infectious disease' was confirmed as a sole or contributory factor to the COD on PME in 41% (21/51) of birds.

The systematic network suspected that 11% of dead birds found over the study period died as a result of 'Infectious disease'. However, the high percentage of birds where the COD was suspected to be 'Predation', or was 'Not established', where PME confirmed 'Infectious disease' involvement, indicates that the importance of infectious disease is likely to be underestimated by the systematic network participants. The percentage of all the dead birds reported by the systematic scheme participants that were suspected to have died due to 'Infectious disease' was re-evaluated on the basis of this appraisal of the network's ability to accurately determine COD. The proportion of birds where

'Infectious disease' was confirmed at PME was calculated for each of the suspected COD categories assigned by the systematic network and then multiplied by the total number of birds in that suspected COD category to provide a crude estimate that c. 41% (1634/4021 birds) of all reported birds died with significant 'Infectious disease'.

3.4 DISCUSSION

Excellent geographical coverage across Great Britain was achieved with both the opportunistic and systematic schemes from the first year, and for subsequent years during the study period, with PME submissions from rural and urban habitats. A small number of regions were underrepresented because of the low human population density and therefore observer effort, for example mid-Wales and northern-Scotland. For the most frequently examined species, PME submissions were received with good coverage across the breeding population range. A longer period of study would be required to achieve comprehensive surveillance across the population range for less frequently submitted species.

Whilst the opportunistic scheme yielded the greatest number of PME submissions, the systematic scheme accounted for c. 40% of submissions from a network of c. 650-750 active participants across the study period, indicating that the size of the recruited participant network was adequate to achieve reasonable balance of submissions between the schemes within the limits of available resource. The first trichomonosis epidemic occurred in summer 2006 (Chapter 6) during year 2 of the study (1st April 2006 – 31st March 2007): the large number of PME submissions with 'Infectious disease' due to this EID from the general public is likely to account for the greater percentage of opportunistic PME submissions in this year. In the third year of study (1st April 2007 – 31st March 2008) systematic submissions were prioritised due to limited financial resources, particularly during the 2007 trichomonosis epidemic: consequently resource management rather than bird mortality drivers may explain the trend toward increased systematic submissions in that year.

This study found no significant difference among the schemes in the number of birds submitted for PME for many of the avian families. However, Fringillidae submissions were more frequent in the opportunistic scheme and Turdidae more frequent in the systematic scheme. The explanation for this appears most likely to relate to COD category: 'Infectious disease' was the most common COD category confirmed on PME for Fringillidae cases whilst 'Other trauma' was the most common for Turdidae (Chapter 4): opportunistic reporters were more likely to submit bird carcasses for PME where an infectious disease was suspected and systematic participants were specifically requested to submit birds of all COD categories, including 'Other trauma' birds such as window collision cases (see below). Similarly, the increase in opportunistic submissions that occurred in the autumn might be attributed to the epidemic trichomonosis mortality which occurred in 2006 and 2007, peaking in September of each year (Chapter 7).

Members of the general public are most likely to be motivated to opportunistically report garden bird mortality, and submit a carcass for PME, if they have observed morbidity or mortality of multiple birds. These events raise public concern for several reasons including animal welfare, potential human, companion animal or livestock health implications or environmental intoxication. Consequently, it is not unexpected that a greater proportion of birds submitted for PME through the opportunistic than systematic scheme died as a result of 'Infectious disease'.

Opportunistic submission of individual birds that are suspected to have died as a result of 'Predation', or 'Other trauma' such as window collision, is considered unlikely by the author unless the affected species is particularly charismatic or known to be of conservation concern. In contrast, systematic scheme participants were requested to submit birds of all COD categories to facilitate collection of baseline data and tissue samples from 'Other trauma' cases to provide a 'control' dataset. Consequently a greater proportion of 'Other trauma' and 'Predation' cases were submitted in the systematic than opportunistic dataset. This underlines the importance of a thorough understanding of the method of surveillance, and carcass selection criteria, to appraise the data

collected: caution should be exercised in extrapolating COD findings from the study population to the national population.

Mortality of in excess of 4,000 dead birds involving a diverse range of species was reported by the systematic participants over the study period: this represents a tremendous information resource that could not realistically be generated without adopting a citizen science approach. Whilst systematic scheme participants were requested to submit birds of all suspected COD and all species throughout the year, submission biases were confirmed.

Systematic participants were most likely to submit birds where the suspected COD category was 'Infectious disease' or 'Not established': it is likely that in these cases the participant had the greatest personal interest in submitting the bird for PME since they were interested to understand the cause of the mortality and they felt that the carcass had greatest potential value to the study. Birds suspected to have died of 'Other trauma' were typically observed to be window collision or road traffic accident injury cases and, since the COD was 'known' the carcasses, although freshly dead and intact, might have been less frequently submitted. Carcasses of birds suspected to have died of 'Predation' may have been in an unsuitable state for PME, for instance partially eaten or scavenged.

The avian families for which dead birds were most likely to be submitted if found, included the Picidae, Fringillidae and Prunellidae. Woodpecker species are highly charismatic with their bright and colourful plumage consequently motivation to submit for PME is likely to be high. Fringillidae are commonly affected by 'Infectious disease' and bias towards this COD category is likely to account for the high rate of submission for PME (Chapter 4). Dunnocks (*Prunella modularis*), however, are cryptic, small and infrequently affected by 'Infectious disease' (Chapter 4): the reason why this species should have a high submission rate for PME is unclear. Avian families which were less likely to be submitted for PME included the Phasianidae, Troglodytidae and Columbidae. Pheasants (*Phasianus colchichus*) are large in body size which would complicate postal submission and increase its cost; game birds are also perceived as

semi-captive and since they are not included within a traditional definition of garden birds they might have been considered outside the main scope of study by participants. The wren (*Troglodytes troglodytes*) is small in body size with cryptic plumage therefore they might be found in a more advanced state of decomposition making submission for PME less likely. Columbidae are also relatively large in body size: feral pigeons are considered a pest species and may be less frequently submitted for PME as a consequence.

It is possible that more rapid rates of decomposition that occur during the warm summer months might explain the reduced proportion of carcasses that were submitted during that season since they were in an unsuitable state for PME. A follow up survey of systematic participants could be performed to determine the factors that influenced whether they opted to submit a bird carcass for examination.

Predation of wild birds by domestic cats (*Felis catus*), the most numerous mammalian predator species in Great Britain, may adversely impact native avian populations (May 1988; Baker et al., 2008). Woods et al. (2003) extrapolated the findings of their study and estimated that a British population of approximately 9 million cats brought home a total of 25-29 million wild birds as prey items over a 5-month period in 1997. Determining whether cat predation represents compensatory or additive mortality is crucial in assessing its impact at the population level. It has been postulated that birds falling prey to predators may include a high number of inexperienced juveniles or individuals of poor health status when compared with non-prey conspecifics. Baker et al. (2008) found significantly poorer body condition and lower body weight in wild birds that died as a result of cat predation than collision injury, the latter taken to represent a random control sample of the population. A multi-species passerine study measured spleen weight as an indirect measure of immunocompetence and found that prey individuals had significantly smaller spleens than non-prey conspecifics suggesting that they had compromised immune systems (Møller et al., 2000).

Infectious disease predisposing to predation was detected in a considerable proportion of cat predation cases in this study. This finding supports the hypothesis that cats favour compromised prey with poor long-term survival prospects. An increased frequency in the number of prey items presented in the home is frequently an early sign of an outbreak of infectious disease at a feeding station. Passerine predation by raptor species is likely to be influenced by similar factors, with weak or sick birds most vulnerable to attack. This study indicates that the importance of infectious disease predisposing to predation may be significantly underestimated by the general public and that disease investigation should be performed at sites where marked changes in the pattern of observed predation occur.

Systematic network participants were able to identify birds with 'Infectious disease' COD category with high confidence. A high proportion of cases where the COD category was 'Not established' at submission were accounted for by 'Infectious disease' confirmed at PME. This is the first time a study has been able to evaluate the ability of a citizen science network to accurately confirm COD categories for wildlife mortality through comparing suspected COD with the results of PME. This enabled us to interrogate the entire dataset from the systematic network, of over 4,000 dead bird reports, with increased confidence: collectively, these findings indicate that 'Infectious disease' is likely to be a major cause of mortality of the garden birds reported dead in this study (crude estimate of c. 40% of dead bird reports). Consequently an understanding of endemic and emerging disease threats of British birds should be an integral component of any conservation or management strategy for avian species that utilise garden habitats.

How accurately the breakdown of COD categories reported in this study reflects actual COD categories of British birds within garden habitats is difficult to evaluate and likely to vary with site, species complement and local habitat type. Birds affected by some infectious diseases frequently congregate at feeding stations and remain immobile with fluffed up plumage: consequently they are highly visible. Starvation and hypothermia, following exposure, were relatively infrequent COD categories in this study. It is

plausible that the carcasses of birds that die of these causes remain hidden in vegetation, or less visible areas of the garden, where they are quickly removed by scavengers before being found.

Neither is it possible to generate an accurate estimate of total bird mortality at systematically monitored gardens through extrapolation from the number of dead birds reported in this study; however, it can be presumed that considerable underreporting occurred. A number of factors are likely to influence the proportion of dead birds reported, including observer vigilance, local habitat type and density and the frequency of monitoring. Wobeser et al. (1992) placed 50 day old chicks at random locations in a mixed-grass pasture in Canada at a density of fifty birds per hectare each day for 5 days. A systematic search of 10% of the area retrieved 20% of birds 24 hours after placement implying that the remainder had been removed by local scavengers. Seasonal differences in scavenger activity, and rates of carcass decomposition, were suggested as explanations for variation in the duration of persistence of song bird carcasses placed in fruit orchards in the USA (Tobin 1990). Balcomb (1986) studied rates of bird carcass removal in agricultural fields in the USA and found that the majority of birds scavenged were removed without residual evidence (e.g. feather remains) and that the relative importance of diurnal and nocturnal scavengers was similar. Ward et al. (2006) used decoy studies with American crows (*Corvus brachyrhynchos*) and house sparrows and found that decoy detection was greater in urban than rural environments in the USA, consequently human population density and related factors could influence the perceived distribution of a disease causing wild bird mortality monitored through opportunistic surveillance. Carcass persistence averaged 1.6 days in rural and 2.1 days in urban areas and was greater for the larger species' carcasses (Ward et al., 2006). Whilst inter-site variation in the intensity of surveillance is likely between systematically monitored sites, all participants in this study were asked to maintain relatively constant observation effort across reporting weeks at each site (Cannon et al., 2005).

The GBH*i* model benefits from the advantages and minimises the disadvantages of both reporting schemes. The large potential observer effort for the opportunistic scheme offers the greatest opportunity to identify novel reports in contrast to the comparably small systematic network. However the quality and depth of reports received is greater from the motivated and experienced GBW network (systematic submissions) than from the general public (opportunistic submissions). The systematic surveillance can be used to verify findings from the opportunistic surveillance scheme, for example temporal or spatial reporting trends that could be influenced by regional media (Eidson et al., 2001) (Chapter 7).

In conclusion, the GBH*i* model with 2 independent yet complementary schemes offers an optimal approach for surveillance of garden bird mortality across Great Britain. This was achieved in a cost-effective manner through collaboration between ornithologists and veterinarians, non-governmental organisations, government agencies and garden bird food companies maximising output from the existing infrastructure and expertise. Constant information exchange, with regular mailing updates to the systematic participant network (Appendix 6&7), and feedback of PME results to all submitters, is vital to ensure continued compliance and participation in the scheme. The GBH*i* model could be used for disease investigation of other wildlife species with small body size and favourable public perception which utilise garden habitats in the future. However, it should be recognised that surveillance schemes that rely on reporting of mortality events may fail to detect pathogens that result in subclinical disease or that lead to altered behaviour patterns where affected birds become increasingly cryptic, rather than highly visible at garden feeding stations. Live bird surveillance schemes, for example in liaison with licensed ringers, may be appropriate for detection of a greater range of pathogens with spectrum of effects ranging from asymptomatic infection to clinical disease.

CHAPTER 4: SUMMARY OF POST MORTEM EXAMINATION FINDINGS ON BRITISH GARDEN BIRDS, 2005 TO 2008

4.1 INTRODUCTION

There have been no recent comprehensive surveys of the infectious or non-infectious diseases of garden bird species in Great Britain. The last multi-species reviews were published by Jennings (1954), Keymer (1958), and Baker (1977): these authors found a broadly similar range of infectious diseases affecting passerine species including avian pox infection, mycobacteriosis, pasteurellosis, salmonellosis, trichomonosis and yersiniosis, amongst others. More recently, Pennycott et al. (1998a) reviewed the causes of mortality in Fringillidae species from Scotland and found infectious disease to be a common cause of death (COD), chiefly salmonellosis due to *S.* Typhimurium definitive type (DT)40 and DT56 variant(v) and colibacillosis due to *E. coli* serotype O86.

Minimal research has been performed to compare the causes of death between bird families and the pathogens that typically affect each group in Great Britain. Jennings (1961) reviewed post mortem examinations (PME) performed on over 1000 wild birds in Britain and found that trauma was the chief COD, accounting for the majority of Turdidae, Fringillidae and Passeridae species examined. Parasites and organic disease were also diagnosed in Columbidae and Fringillidae species.

This chapter presents the findings of PMEs performed on 1,559 garden birds of 49 bird species across Great Britain over a 3-year period. This represents the most extensive overview of infectious disease in native garden birds in the past 30 years. COD categories were summarised for all birds examined and compared between avian families. The most frequently diagnosed infectious diseases considered significant to the COD were investigated in detail. Unusual mortality events classified as 'Other' COD were also described.

4.2 MATERIALS AND METHODS

Case selection and submission

PMEs were performed on carcasses submitted from a subset of reported garden bird mortality incidents using a standardised protocol (Appendix 5). Carcasses from Scotland were submitted to the Disease Surveillance Centre Scottish Agricultural College (SAC); from Cheshire, Derbyshire, Nottinghamshire, Humberside and more northern English counties were submitted to the Department of Veterinary Pathology, University of Liverpool; from Cornwall to the Wildlife Veterinary Investigation Centre (WVIC) and from the remainder of England and Wales to the Institute of Zoology (IoZ). Data from all PMEs was collated and analysed at the IoZ.

Cases were selected for PME based on fresh carcass availability. Birds thought to have died as a result of all COD categories, including 'Predation', 'Other trauma' and 'Infectious disease' were examined; the selection criteria did not specifically or solely target certain species or suspected cases of infectious disease, but rather aimed to achieve a representative cross-section of species and aetiologies. Carcasses were submitted by post observing packaging guidelines (Appendix 2) or were hand-delivered. Carcasses were refrigerated at 4 °C on arrival and examined fresh within 48 hours of submission where possible, or were frozen at -20 °C on submission and examined at a later date. The majority of birds were found dead by members of the public; a small number of birds (c. 2%) were euthanased on welfare grounds using chemical or physical techniques.

Gross examination

Each submitted carcass was assigned a unique reference code and the species, age, sex and body weight were recorded. Birds were classed as nestlings until the primary feathers of the pullus were fully grown and as juveniles until the post-juvenile body moult was complete. First year birds beyond their post-juvenile moult and adult birds were not differentiated; all were classified as adult for the purpose of this study. Sex was assigned based on gonadal inspection and/ or plumage characteristics. The state of

carcass preservation was described in each case, i.e. freshly dead, mild, moderate or advanced degree of autolysis. Systematic examination of the external features (skin, plumage, orifices, uropygial gland, legs and feet) was performed and details of moult were described when present. A subjective measure of body condition ('emaciated', 'thin', 'moderate' or 'good') was made based on visual inspection of the pectoral muscle and fat deposits. Biometric measurements including the maximum tarsus length (to the nearest 0.1 mm) and wing length (to the nearest mm) were recorded at the IoZ and WVIC, using techniques published by Redfern et al. (2001). Systematic internal examination of the body systems was performed and any gross lesions described. At the IoZ and WVIC, both superficial pectoral muscles and the liver were dissected and weighed (to the nearest 0.1 gram). At the IoZ, photographs were routinely taken for species identification, of lesions and other noteworthy findings. Radiographs were taken at the Zoological Society of London veterinary hospital in a small number of cases to assess skeletal mineralization.

Where indicated, and where the degree of carcass decomposition permitted, samples were taken for microbiological, parasitological and histopathological investigations.

Microbiology

Liver and contents of the mid-small intestinal loop were sampled aseptically from the majority of cases, as were any lesions found, and were examined for the presence of pathogenic bacteria. Briefly, liver was plated directly onto the following media: Colombia blood agar supplemented with 5% horse or sheep blood (CBA) (QCM laboratories or E and O laboratories), incubated under aerobic conditions, and Chocolate blood agar (CCBA) (QCM laboratories), incubated under 5-10% CO_2 conditions. Small intestinal contents were plated directly onto the following media: (1) Xylose-Lysine Deoxycholate (XLD) agar (QCM laboratories), or MacConkey agar without salt (E&O laboratories) and Brilliant green agar (E&O laboratories), incubated under aerobic conditions; (2) CBA, incubated under aerobic conditions; (3) Campylobacter Blood Free Selective medium (modified CCDA-Preston) (QCM laboratories), incubated under microaerophilic conditions, and (4) immersed into selenite Salmonella-selective

enrichment broth (QCM laboratories or E&O laboratories) under aerobic conditions for 24 h followed by subculture onto XLD agar aerobically. At the IoZ, liver also was plated onto CBA and incubated anaerobically.

Heart blood was cultured in a minority of cases using the same protocol as the liver. Lung was routinely cultured from all Paridae and Aegithalidae submissions at the IoZ using the same protocol as the liver. The same bacteriology protocol was used for examination of necrotic ingluvitis lesions as for the intestinal contents with the exception of the modified CCDA-Preston media. All culture media were incubated at 37 °C with the exception of selenite broth at the SAC, which was incubated at 42 °C.

Bacterial isolates were identified using colony and Gram's staining morphology, followed by biochemical properties which were determined using the Analytical Profile Index (API) biochemical test strip method (API-BioMerieux, Marcy l'Etoile, France). *Yersinia enterocolitica, Yersinia pseudotuberculosis, Pasteurella/ Pasteurella multocida* and *Campylobacter* spp. were identified on this basis alone. Slide agglutination tests were performed for the identification of suspected *Salmonella* spp. isolates using poly-O antisera (Pro-lab diagnostics). Bacterial isolates for which further typing was required were placed onto microbank beads (Pro-lab diagnostics) and stored at both –25 °C and –70 °C.

Batches of *Salmonella* spp. isolates from cases in England and Wales were submitted to the Salmonella Reference Unit, Health Protection Agency (HPA) for biotyping (serotype and phage type) according to standardised international protocols (Anderson et al., 1977). *Salmonella* spp. isolates from Scotland were submitted to the Scottish Salmonella Reference Laboratory at Stobhill for biotyping. Isolates that did not correspond to established phage types were classified as 'reacts does not conform' (RDNC).

Non-lactose or late lactose fermenting *E. coli* isolates cultured from small intestinal contents were submitted to the SAC where they were serotyped using micro-

agglutination and slide tests with antiserum against *E. coli* serotype O86 (raised in rabbits at the Central Veterinary Laboratory) (Foster et al., 1998).

Suttonella ornithocola isolates were identified by their morphology (Gram-negative rod to coccobacillus, 0.5-0.6 x 0.6-1.3 μm), preference for aerobic and capnophilic (or carboxyphilic) culture conditions, oxidase positive reaction and biochemical test results (API 20NE, bioMérieux), as described by Kirkwood et al. (2006). Isolated were submitted to the HPA where the 16S rRNA genes were amplified by PCR using universal primers pA and sequenced to confirm the isolate identity as *S. ornithocola* (Foster et al., 2005).

In addition to routine bacteriological examinations, crop and lung lesions, and other lesions where a mycotic causative agent was suspected, were plated on to Sabourauds agar (QCM laboratories) for the isolation of fungal species. Isolates, such as *Aspergillus fumigatus* were identified in-house from morphological characteristics, while other fungi, such as *Candida albicans*, were identified in-house using the API-32C (bioMérieux) test kit for the biochemical identification of yeast species or by germ tube formation.

Parasitology

Ectoparasites and metazoan endoparasites were examined under the dissecting microscope to facilitate identification and fixed directly in 70% ethanol. Wet mount preparations of small intestinal contents were examined in a subset of cases for evidence of nematode, cestode and protozoan parasites. Protozoal oocysts were archived in 2% aqueous potassium dichromate solution and stored at 4 °C.

Ixodid ticks were submitted to the Medical Entomology & Zoonoses Ecology group, HPA, for species identification. A subset of nematode parasites was submitted to Dr E. Harris at the Parasitic Worms Group, Natural History Museum, for species identification. A subset of coccidial oocysts was submitted to Professor S.J. Ball for protozoal species identification.

Oesophageal lesions (c. 5mm^3) from cases with necrotic ingluvitis, in mild states of autolysis, were incubated at 30 °C in Trichomonas Media No. 2. (Oxoid, UK) and screened for motile trichomonads at 24, 48, 72 hrs and 5 days. In addition, saline-mounted wet preparations were prepared from some freshly dead carcasses with necrotic ingluvitis and examined for motile trichomonads. Nested PCR developed from the trichomonad small subunit rRNA primers used by Cepicka et al. (2005) was used for trichomonosis case diagnosis (Chapter 6).

Histopathology

Where the state of carcass preservation permitted, samples from a range of organs (including brain, gizzard, heart, kidney, liver, lung, pectoral muscle, small intestine, spleen, trachea and any diseased tissues), including any lesions found, were fixed in neutral-buffered 10% formalin and processed for histopathological examination using routine methods. Tissue sections were stained using Haematoxylin and Eosin as standard (Abbey Veterinary Services, UK). Slides from cases suspected to be *Suttonella ornithocola* infection were stained in addition with Gram-Twort. Slides from suspected mycobacteriosis cases were stained with Ziehl-Neelsen.

Transmission electron microscopy was performed on suspected avian pox skin lesions that were fixed in 2.5% buffered gluteraldehyde and post-fixed in 1% osmium tetroxide (VWR, UK) at the University College Medical School, Royal Free Campus, using Philips 201 and 501 microscopes.

Sample archive

Samples of organs and diseased tissues were stored frozen at -20 °C or -80 °C for future analyses. Details of the sample archive were recorded on the PME records (Appendix 5).

Cause of death categories

A COD category was assigned for each case following review of the pathological examinations performed. Categories included 'Infectious disease', 'Predation', 'Other trauma', 'Other' and ''Not established'. For the purposes of this chapter, the 'Infectious

disease' category includes cases which were considered to have died as a result of infectious disease alone, or where infectious disease was thought to be an important contributory factor to the COD in combination with another factor (e.g. 'Other trauma' or 'Predation'). The 'Other trauma' and 'Predation' categories represent cases where they were considered to be the sole COD and no evidence of other significant abnormalities was found. The 'Other' category includes miscellaneous conditions such as starvation, exposure and euthanasia. The results of PME from opportunistic and systematic submissions are combined in this chapter.

The breakdown of COD categories was compared between the most commonly examined avian families (Columbidae, Fringillidae, Paridae, Passeridae, Turdidae) and among species within each family. The number of cases (and sites) from which infectious diseases were diagnosed was summarised by aetiology including bacterial (avian tuberculosis, colibacillosis, pasteurellosis, *Suttonella ornithocola* infection, yersiniosis), fungal (aspergillosis), viral (avian pox infection) and parasitic (coccidiosis, helminthosis, trichomonosis) conditions. Diagnosis was reached on the basis of pathogen isolation in combination with gross or histopathological lesions characteristic for the infectious disease. In addition, the following case definitions were employed:

Avian pox infection was confirmed on the basis of either histopathology (presence of pathognomonic intacytoplasmic inclusion Bollinger bodies (Ritchie 1995)) or transmission electron microscopy (presence of virions of characteristic shape and profile) of skin lesions. Suspected cases of avian pox infection had typical skin lesions where PME yielded no evidence of alternative aetiology (bacterial or parasitic) but carcass condition precluded further examination.

Colibacillosis with *E. coli* serotype O86 infection was confirmed on the basis of a non-lactose fermenting or late lactose fermenting *E. coli* isolate cultured from the small intestinal contents with a characteristic API 20E profile (4144102 and 5144102) confirmed to be seropositive by the SAC, coupled with signs consistent with colibacillosis such as an empty gastrointestinal tract or discoloured intestinal contents.

Salmonellosis was confirmed on the basis of characteristic gross lesions and isolation of *Salmonella* spp. on direct culture in pure/ heavy growth from site(s). Suspected salmonellosis cases were defined as those with isolation of *Salmonella* spp on direct culture in pure or heavy growth, in poor body condition but with no gross lesions where bacterial septicaemia, or microscopic salmonellosis lesions, may have been present but could not be confirmed. Suspected *Salmonella* sp. carriers were defined as those with isolation of a *Salmonella* sp. on culture from site(s) (on direct culture or following selenite enrichment only), with no gross lesions and good body condition, where an alternate COD was confirmed.

Trichomonosis was confirmed on the basis of necrotic ingluvitis lesions in combination with culture of motile trichomonads and/ or positive nested PCR amplification (Chapter 6). Cases with characteristic gross lesions from which a *Salmonella* sp. (the most common differential diagnosis for necrotic ingluvitis in passerines) could not be isolated, but in which confirmation of trichomonosis through culture or nested PCR was not possible, were considered 'suspected trichomonosis'.

Each of the most commonly diagnosed infectious diseases were then reviewed in detail, focusing on features most relevant to each condition including the species affected, case demography, lesions observed, pathogen typing, spatial and temporal trends in occurrence and number of birds affected per incident. Pathogens that were detected but considered incidental to the COD were not presented in this chapter.

Non-parametric analyses including the Pearson chi-squared test and binomial test of proportions were performed as appropriate, unless otherwise indicated, using SPSS 17.0 for Windows (SPSS inc., Chicago, USA) and R--CRAN (http://www.R-project.org). Spatial data were presented using ArcView 3.0 geographical information system (GIS) software (Environmental Systems Research Institute GIS and Mapping Software, California, U.S.A.).

4.3 RESULTS

Species breakdown

Some 1,559 PMEs were performed across all centres between 1st April 2005 and 31st March 2008 (Table 4.1). These included 929 bird submitted opportunistically by members of the public and 630 submitted systematically through the BTO Garden BirdWatch network. Greenfinches (n=539), chaffinches (n=276) and house sparrows (n=109) were the most frequently examined species in rank order. A total of 48 species was examined: Accipitriformes (Accipitridae (n=9)); Columbiformes (Columbidae (n=88)); Ciconiiformes (Ardeidae (n=1)); Galliformes (Phasianidae (n=1)); Passeriformes (Aegithalidae (n=5), Corvidae (n=10), Emberezidae (n=12), Fringillidae (n=1004), Hirundidae (n=3), Motacillidae (n=1), Muscicapidae (n=1), Paridae (n=61), Passeridae (n=118), Prunellidae (n=42), Regulidae (n=4), Sittidae (n=3), Sturnidae (n=31), Syliviidae (n=9), Troglodytidae (n=2), Turdidae (n=137)); Piciformes (Picidae (n=12)); Psittaciformes (Psitticadae (n=3)) and Strigiformes (Strigidae (n=2)).

Degree of decomposition

The majority of carcasses were examined fresh (90% - 1400/1559), within 48 hours of submission, with the remainder frozen before examination (10% - 159/1559). The state of carcass preservation was recorded as 'freshly dead' in 6% cases (97), 'mild autolysis' in 26% cases (390), 'moderate decomposition' in 54% cases (818) and 'advanced decomposition' in 14% cases (220), of the 1525 cases where details were available.

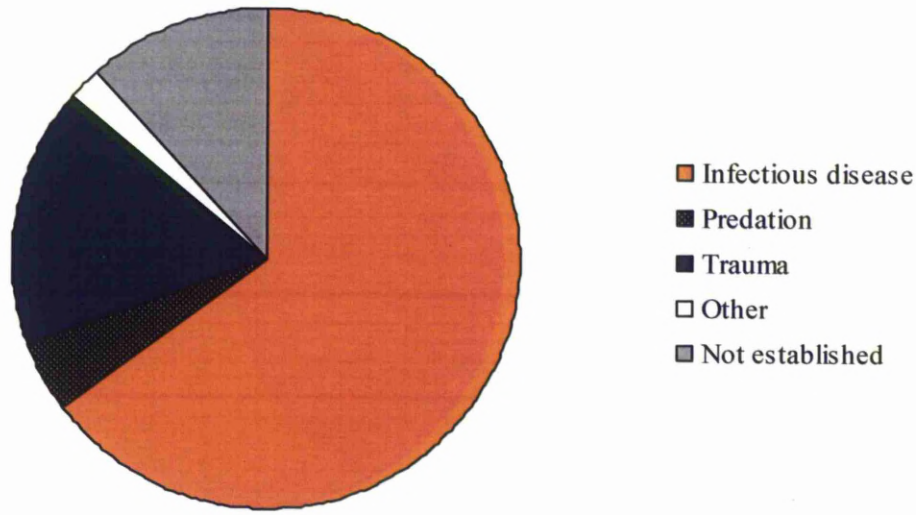
Table 4.1: Species breakdown of PME for all centres (1st April 2005 – 31st March 2008)

Order/ Species	Total	Order/ Species	Total	Order/ Species	Total
Acciptriformes		Reed Bunting *Emberiza schoenicurius*	1	Tree Sparrow *Passer montanus*	9
Common Buzzard *Buteo buteo*	2	Brambling *Fringilla montifringilla*	4	Dunnock *Prunella modularis*	42
Sparrowhawk *Accipiter nisus*	7	Bullfinch *Pyrrhula pyrrhula*	34	Goldcrest *Regulus regulus*	4
Columbiformes		Chaffinch *Fringilla coelebs*	276	Nuthatch *Sitta europea*	3
Collared Dove *Streptopelia decaocto*	41	Crossbill *Loxia curvirostra*	1	Starling *Sturnus vulgaris*	31
Feral Pigeon *Columbia livia*	6	Goldfinch *Carduelis carduelis*	61	Blackcap *Sylvia atricapilla*	8
Wood pigeon *Columba palumbus*	41	Greenfinch *Carduelis chloris*	539	Reed Warbler *Acrocephalus scirpaceus*	1
Ciconiiformes		Hawfinch *Coccothraustes coccothraustes*	1	Wren *Troglodytes troglodytes*	2
Grey Heron *Ardea cinerea*	1	Lesser Redpoll *Carduelis flammea*	2	Blackbird *Turdus merula*	88
Galliformes		Siskin *Carduelis spinus*	86	Mistle Thrush *Turdus viscivorous*	1
Pheasant *Phasianus colchicus*	1	Swallow *Hirundo rustica*	3	Redwing *Turdus iliacus*	5
Passeriformes		Pied Wagtail *Motacilla alba*	1	Robin *Erithacus rubecula*	25
Long-tailed Tit *Aegithalos caudatus*	5	Redstart *Phoenicurus phoenicurus*	1	Song Thrush *Turdus philomelos*	18
Carrion Crow *Corvus corone*	2	Blue tit *Cyanistes caeruleus*	24	**Piciformes**	
Jackdaw *Corvus monedula*	4	Coal Tit *Periparus ater*	7	Green woodpecker *Picus viridus*	2
Magpie *Pica pica*	1	Great tit *Parus major*	29	Great Spotted Woodpecker *Dendrocopus major*	10
Rook *Corvus frugilegus*	3	Marsh Tit *Parus palustris*	1	**Psittaciformes**	
Yellowhammer *Emberiza citrinella*	11	House Sparrow *Passer domesticus*	109	Ring-necked Parakeet *Psittacula krameri*	3
				Strigiformes	
				Tawny Owl *Strix aluco*	2

Cause of death categories

The breakdown of COD categories for all cases examined between 1st April 2005 and 31st March 2008 is shown in Figure 4.1. 'Infectious disease' was the sole COD, or an important contributory factor to death, in the majority of cases (64% - 1011/1559). Predation was considered the sole COD of 5% (70/1559) of cases, 'Other trauma' in 17% (263/1559) of cases and 'Other' causes in 2% (33/1559) of cases. The COD category was 'Not established' for 11% (183/1559) of cases.

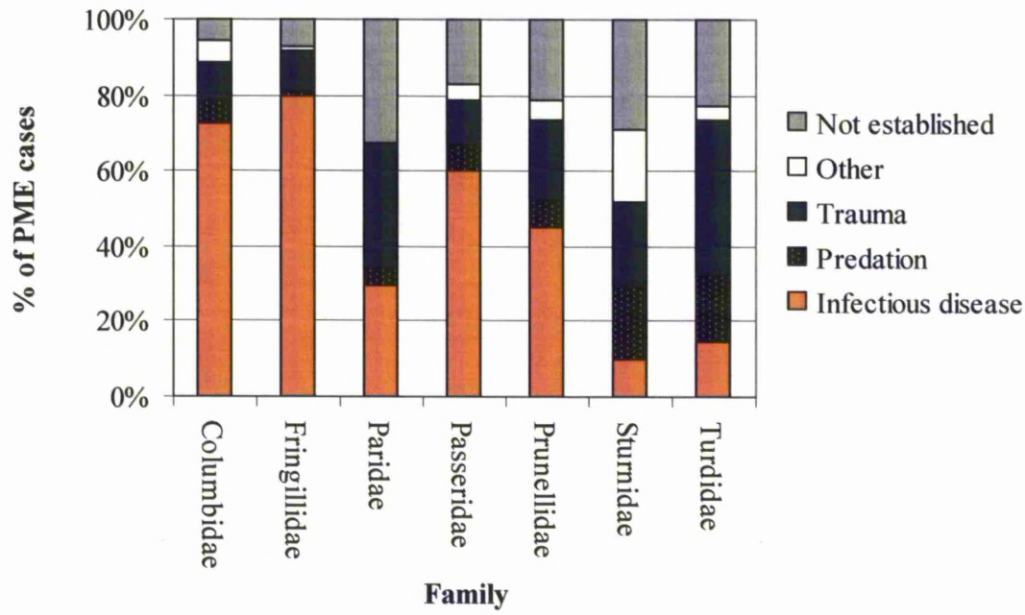
Figure 4.1: COD categories for all PME cases
(1st April 2005 – March 31st 2008).



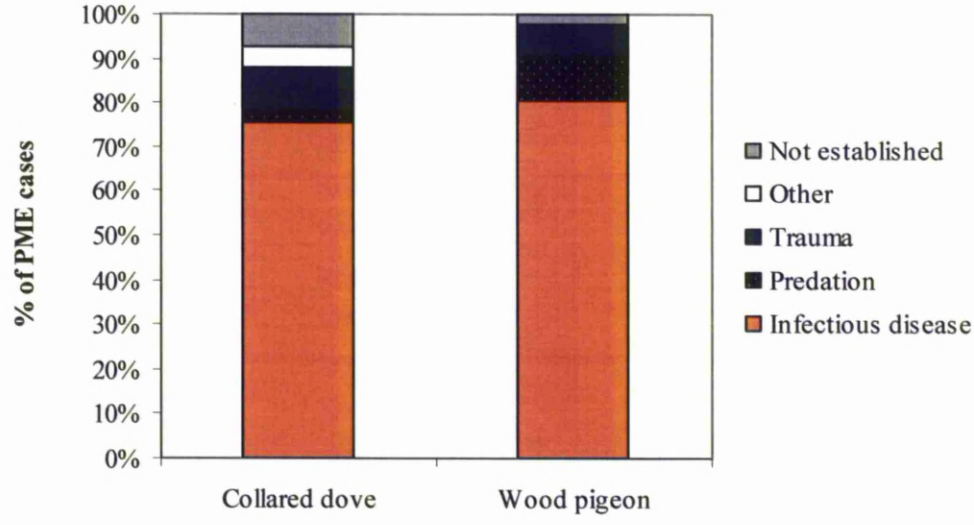
COD categories by avian family and species are presented in Figure 4.2(a-e) for the most commonly submitted bird species examined (1st April 2005 – 31st March 2008).

Figure 4.2: COD categories (a) by Family (b) Columbidae (c) Fringillidae (d) Paridae (e) Turdidae. Species listed individually where 20 or more individuals were examined post mortem.

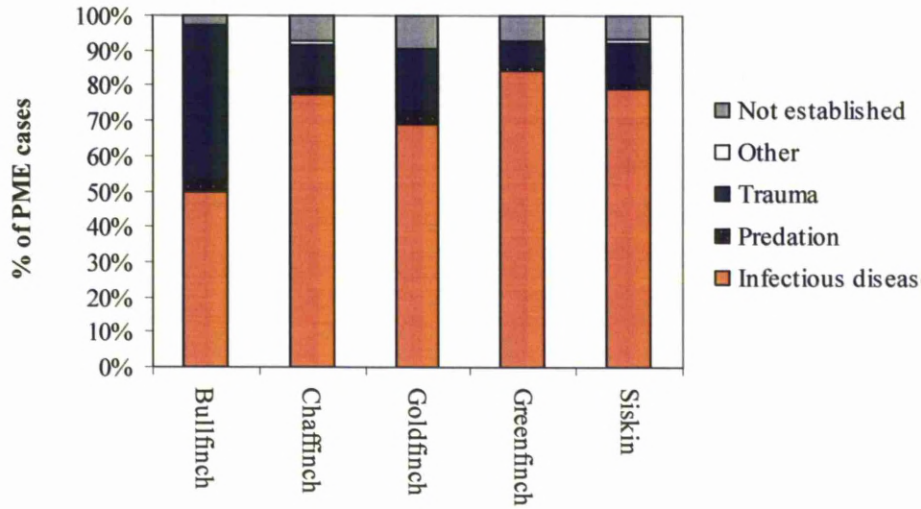
(a) Family (Columbidae (n=88), Fringillidae (n=1004), Paridae (n=61), Passeridae (n=118), Prunellidae (n=42), Sturnidae (n=31), Turdidae (n=137)).



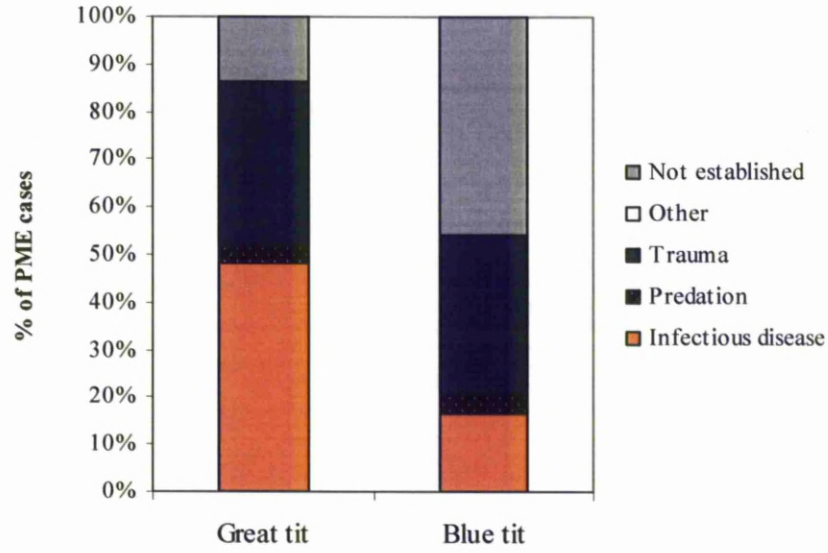
(b) Collared dove (n=41) and Wood pigeon (n=41)



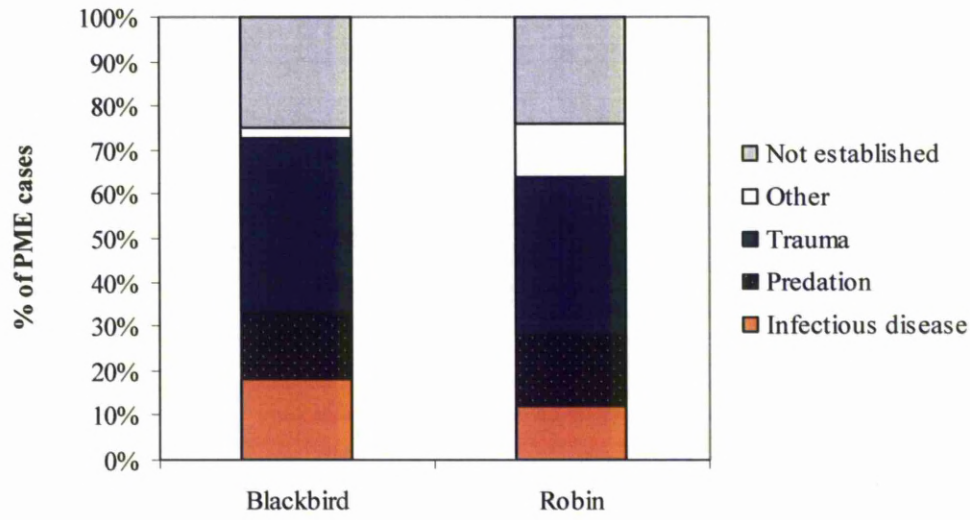
c) Bullfinch (n=34), Chaffinch (n=276), Goldfinch (n=61), Greenfinch (n=539) and Siskin (n=86)



(d) Blue tit (n=24) and Great tit (n=29)



(e) Blackbird (n=88) and Robin (n=25)



Pearson's chi-square test showed that there was a significant difference in the COD categories between the 7 most common bird families (X^2=442.730, df=24, P<0.0001) examined (Figure 4.2a). Infectious disease was the modal COD category for the Fringillidae, Columbidae, Passeridae and Prunellidae, accounting for 80% (800/1004), 73% (64/88), 60% (71/118) and 45% (19/42) of cases respectively.

There was no significant difference in the COD categories between the 2 most frequently examined Columbidae species (Figure 4.2b), the collared dove and wood pigeon (X^2=5.339, df=4, P>0.05) or the 2 most frequently examined Turdidae species (Figure 4.2e), the blackbird and robin (X^2=4.704, df=4, P>0.05).

A significant difference was found between the COD categories of the 5 most frequently examined Fringillidae species (X^2=60.037, df=16, P<0.000) (Figure 4.2c): infectious disease accounted for a significantly smaller percentage of bullfinch cases (50% - 17/34) examined than for the other 4 finch species combined (81% - 777/962) (X^2=17.374, df=1, P<0.0001). If bullfinch was excluded from this group there was no significant

difference in the COD categories for the remaining 4 finch species (X^2 =19.697, df=16, P>0.05).

A significant difference was found between the COD categories of the 2 most frequently examined Paridae species at the 95% confidence level (X^2=8.650, df=3, P=0.03) (Figure 4.2d). Infectious disease accounted for a significantly greater percentage of great tit (48% - 14/29) than blue tit (17% - 4/24) cases (X^2 =4.526, df=1, P=0.03): conversely a significantly smaller percentage of great tit than blue tit cases had the COD 'Not established' (X^2=5.158, df=1, P=0.02).

Table 4.2: Infectious diseases contributing to the COD summarised by avian family
Number of cases (C=confirmed cases/ S=suspected cases; number of sites)
(B) Bacterial (F) Fungal (P) Parasitic (V) Viral aetiologies.

Columbidae

Infectious disease	Collared dove	Feral pigeon	Wood pigeon
Aspergillosis (F)	0	0	1 (1 site)
Avian pox (V)	0	0	1 (1C/0S; 1 site)
Avian tuberculosis (B)	0	0	1 (1C/0S; 1 site)
Pasteurellosis (B)	1 (1 site)	0	0
Trichomonosis (P)	29 (27C/2S; 29 sites)	0	29 (26C/3S; 28 sites)

Fringillidae

Infectious disease	Bullfinch	Chaffinch	Greenfinch	Goldfinch	Siskin	Misc*
Colibacillosis (B)	0	12 (8 sites)	10 (10 sites)	3 (3 sites)	28 (16 sites)	0
Coccidiosis (P)	0	1 (1 site)	22 (22 sites)	1 (1 site)	0	0
Pasteurellosis (B)	0	1 (1 site)	1 (1 site)	0	1 (1 site)	0
Salmonellosis (B)	6 (6 sites)	20 (14 sites)	109 (91 sites)	24 (24 sites)	27 (26 sites)	2 (2 sites)
Trichomonosis (P)	10 (9C/1S; 8 sites)	178 (126C/52S 126 sites)	328 (269C/32S; 263 sites)	13 (11C/2S; 13 sites)	3 (3C/0S; 3 sites)	3 (3C/0S; 3 sites)
Yersiniosis (B)	0	3 (3 sites)	0	2 (2 sites)	0	1 (1 site)

*Brambling, Crossbill, Hawfinch, Redpoll

Paridae

Infectious disease	Blue tit	Coal tit	Great tit
Avian pox (V)	1 (0C/1S;1 site)	0	7 (5C/ 2S; 6 sites)
Pasteurellosis (B)	0	0	1 (1site)
Suttonella ornithocola (B)	4 (4C/0S; 4 sites)	0	0
Trichomonosis (P)	1 (0C/1S; 1 site)	0	4 (4C/0S; 4 sites)

Passeridae

Infectious disease	House sparrow	Tree sparrow
Aspergillosis (F)	1 (1 site)	0
Avian pox (V)	3 (0C/3S; 2 sites)	0
Colibacillosis (B)	0 (0 sites)	0 (0 sites)
Coccidiosis(P)	2 (2 sites)	2 (1site)
Pasteurellosis (B)	3 (3 sites)	0
Salmonellosis (B)	42 (39 sites)	3 (2 sites)
Trichomonosis (P)	10 (8C/2S; 10 sites)	0

Turdidae

Infectious disease	Blackbird	Robin
Aspergillosis (F)	3 (3 sites)	0
Pasteurellosis (B)	2 (2 sites)	2 (2 sites)
Helminthosis (P)	4 (4 sites)	0
Trichomonosis (P)	1 (1 site)	0

Prunellidae

Infectious disease	Dunnock
Avian pox (V)	(1C/3S; 4 sites)
Colibacillosis (B)	1 (1 site)
Coccidiosis (P)	2 (2 sites)
Pasteurellosis (B)	2 (2 sites)
Salmonellosis (B)	2 (2 sites)
Trichomonosis (P)	10 (10C/0S; 10 sites)

Sturnidae

Helminthosis was considered an important contributory factor to the COD of 2 adult starlings from 2 sites. A combination of *Syngamus trachea* and gastrointestinal cestode and nematode parasites (unidentified species) was present in each bird.

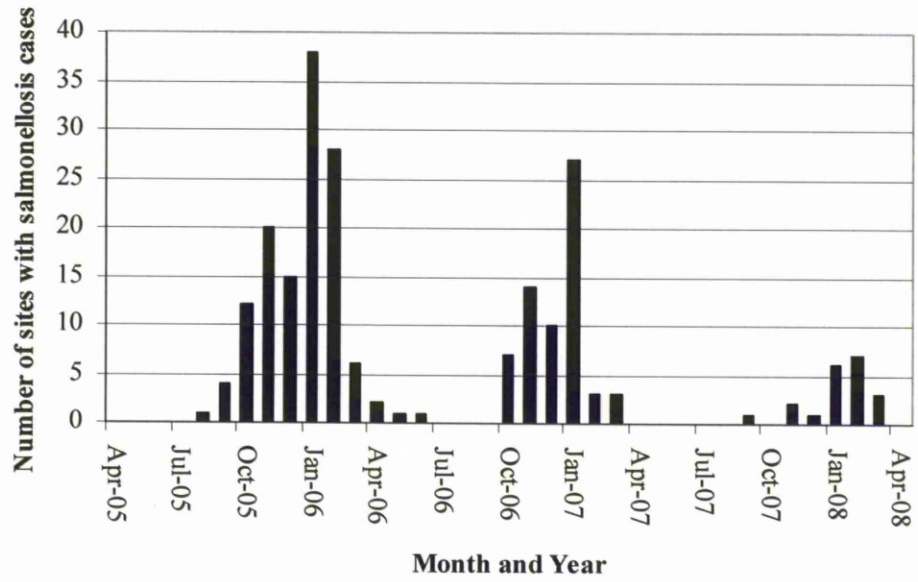
Details of the most frequently diagnosed infectious diseases considered significant to the COD of birds examined are reviewed for bacterial, fungal, parasitic and viral pathogens in turn, in rank order from the most to the least common for each pathogen group.

BACTERIAL

Salmonellosis

Salmonellosis was diagnosed as the COD in a total of 251 cases from 179 sites across Great Britain between 1st April 2005 and 31st March 2008. Multiple focal lesions were typically observed in the upper alimentary tract, liver and spleen, coupled with hepatomegaly and splenomegaly in some salmonellosis cases (Chapter 5). Salmonellosis was confirmed most frequently in male greenfinches and the sex bias was significant when compared with a theoretical population with a sex ratio of unity (63 males, 35 females; X^2=7.439, df=1, P=0.006). There was no similar skew in the sex of house sparrow cases (23 males, 17 females; X^2=0.625, df=1, P>0.05). Salmonellosis incidents were highly seasonal, principally during the winter months, with 98% (245/251) of cases at 98% (176/179) of sites between September and March and a peak number of 37% (93/251) of cases at 36% (65/179) of sites in January. The number of salmonellosis incidents received by salmonellosis season (September to March inclusive) reduced year on year during the study period: salmonellosis incidents from 109 sites in September 2005 - March 2006; from 60 sites in September 2006 - March 2007; and from 18 sites in September 2007 - March 2008 (Figure 4.3). The decline was observed in salmonellosis cases submitted through both the opportunistic and systematic surveillance schemes. Salmonellosis cases were confirmed in multiple salmonellosis seasons at 11 sites: 10 sites had confirmed cases in 2 salmonellosis seasons and a single site had cases confirmed in each salmonellosis season during the 3-year study period.

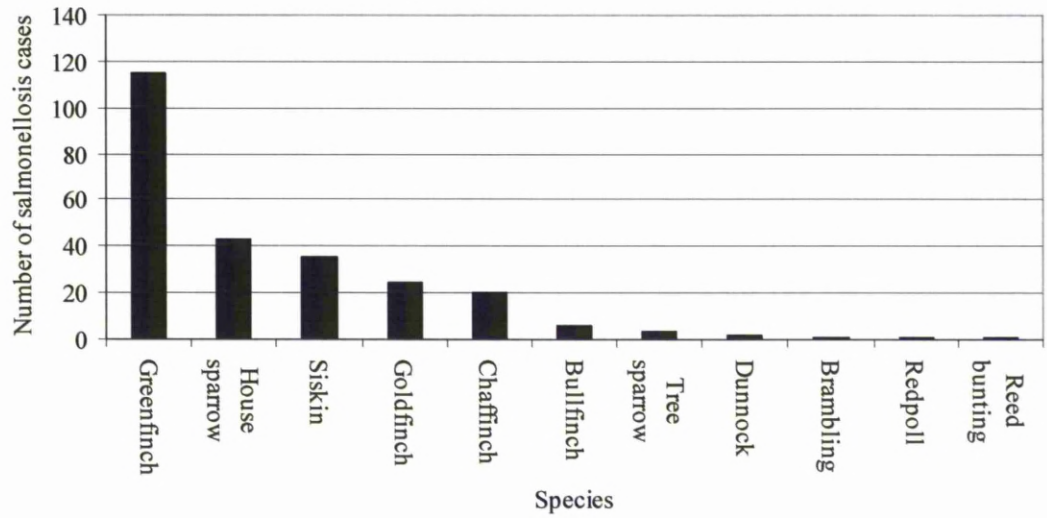
Figure 4.3: Number of sites with salmonellosis incidents by month (1st April 2005 – 31st March 2008).



Salmonellosis was confirmed in birds of 11 species, principally Fringillidae (80% - 202/251 cases) and Passeridae (18% - 46/251 cases), with isolated cases in Emberizidae (1% - 1/251 cases) and Prunellidae (1% - 2/251 cases) species. The overall species breakdown of salmonellosis cases by species is shown in Figure 4.4.

Contemporaneous morbidity or mortality of greenfinches was recorded at 54% (90/168) of the sites where salmonellosis was confirmed in a single season (September to March inclusive); no sign of ill health was recorded in greenfinches at 44% (74/168) of these sites and 4 sites were data deficient. For the 11 sites with salmonellosis confirmed in birds on multiple seasons throughout the 3-year study period, 4 sites had contemporaneous greenfinch morbidity or mortality, 4 had no observed evidence of greenfinch disease and 3 sites had greenfinch involvement in salmonellosis incidents in one but not all seasons where cases occurred.

Figure 4.4: Species breakdown of salmonellosis cases (1st April 2005 – 31st March 2008).

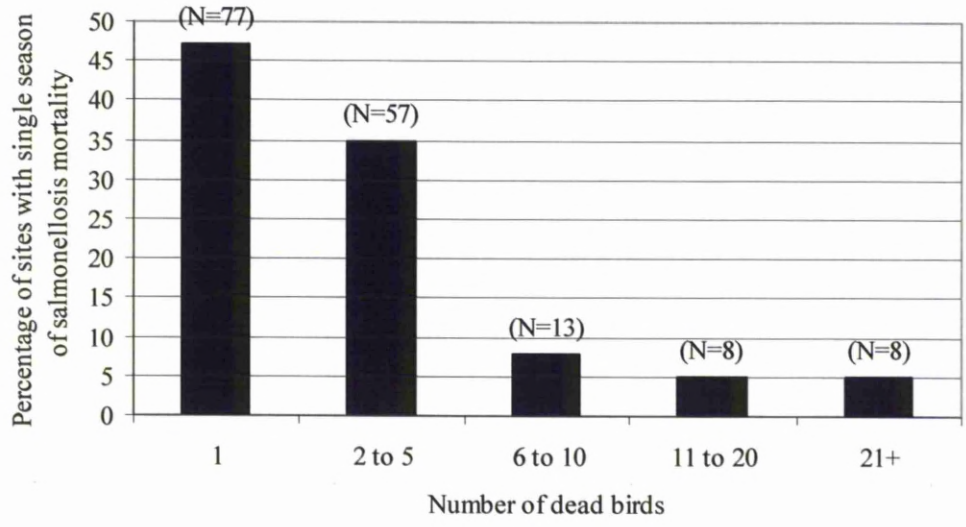


For sites where salmonellosis was confirmed in less frequently affected species (i.e. bullfinch, brambling, dunnock, and tree sparrow), the history was reviewed for evidence of contemporaneous greenfinch morbidity or mortality: possible evidence of diseased greenfinch(es) was noted in 1 of 6 sites with affected bullfinches, 1 of 2 sites with affected dunnocks and 1 of 2 sites with affected tree sparrows. Concurrent morbidity or mortality of siskins was seen at the site where the brambling was confirmed with salmonellosis. A single species only was seen to be affected at 5 of the 6 sites with affected bullfinches, 1 of 2 sites with affected dunnocks, 1 of 2 sites with affected tree sparrows and the sites where salmonellosis was confirmed in a reed bunting and redpoll.

The total number of dead birds (of the 11 species in which salmonellosis was confirmed at PME) reported by site ranged from 1-130 (mean=4.63, SEM=0.81); of those sites with salmonellosis reported during a single season, 46% (77/168) had only a single death reported and 38% (57/168) had 2-5 dead birds (Figure 4.5). All salmonellosis incidents

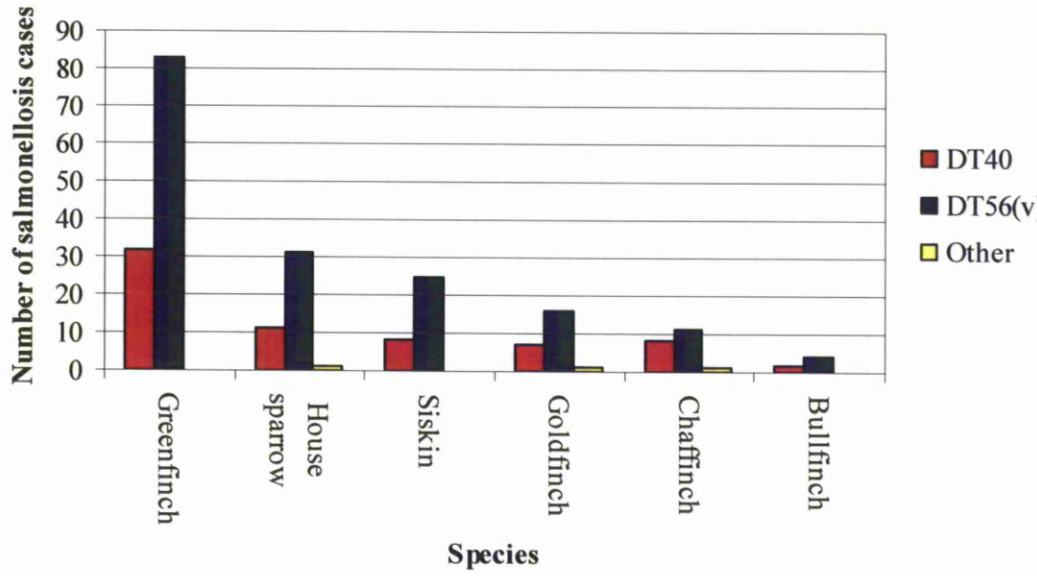
from sites with cases confirmed in multiple seasons reported less than 5 dead birds with a single exception where in excess of 20 dead birds were found.

Figure 4.5: Number of dead birds reported at each site with confirmed salmonellosis in 1 or more birds at PME (1st April 2005 – 31st March 2008).



Salmonella Typhimurium phage typing confirmed that isolates from 71% (177/251) of cases were DT56(v) and 27% (69/251) of cases were DT40. Single cases of salmonellosis were recorded with isolates of the following phage types: a goldfinch with DT81, a chaffinch with DT193 and a house Sparrow with U277. No cases of *S.* Typhimurium DT160, a previously common phage type, were observed in this study period. No significant difference was found in the proportion of salmonellosis cases from which *S.* Typhimurium DT40 and DT56(v) was isolated in the 5 species most frequently affected (X^2=2.199, df=4, P>0.05) (Figure 4.6).

Figure 4.6: Number of salmonellosis cases with each *S.* Typhimurium phage type by species (1st April 2005 – 31st March 2008).



Salmonellosis was confirmed at sites widespread across Scotland and Wales; the regions with low reporting rates correspond to areas of low human population density (Figure 4.7). In England, salmonellosis occurred principally in the western counties with very few incidents confirmed in the central and eastern counties, although birds were examined from across these regions (Appendix 8). Salmonellosis cases were confirmed from sites across Great Britain for both the greenfinch and house sparrow with similar distribution. Salmonellosis cases were confirmed from sites across Great Britain in the first 2 seasons (September 2005 to March 2006, and September 2006 to March 2007) (Figure 4.8). In contrast, by year 3 (September 2007 to March 2008) when the number of salmonellosis incidents was considerably reduced, the majority (78% - 14/18) of affected sites were in Scotland (Figure 4.8c). The distribution of salmonellosis cases with each of the most common *S.* Typhimurium phage types, DT40 and DT56(v), varied with region (Figure 4.9). *S.* Typhimurium DT56(v), the predominant phage type, was confirmed from incidents across Great Britain but *S.* Typhimurium DT40 was confirmed

from incidents principally restricted to Scotland and south-west England, although some incidents occurred in the Welsh Borders.

Figure 4.7: Distribution of confirmed cases of salmonellosis (1st April 2005 – 31st March 2008) in (a) all species (b) greenfinch and (c) house sparrow.

(a)

(b)

(c)

Figure 4.8: Distribution of all confirmed cases of salmonellosis in all species in (a) September 2005 – March 2006 (b) September 2006 – March 2007 and (c) September 2007 – March 2008.

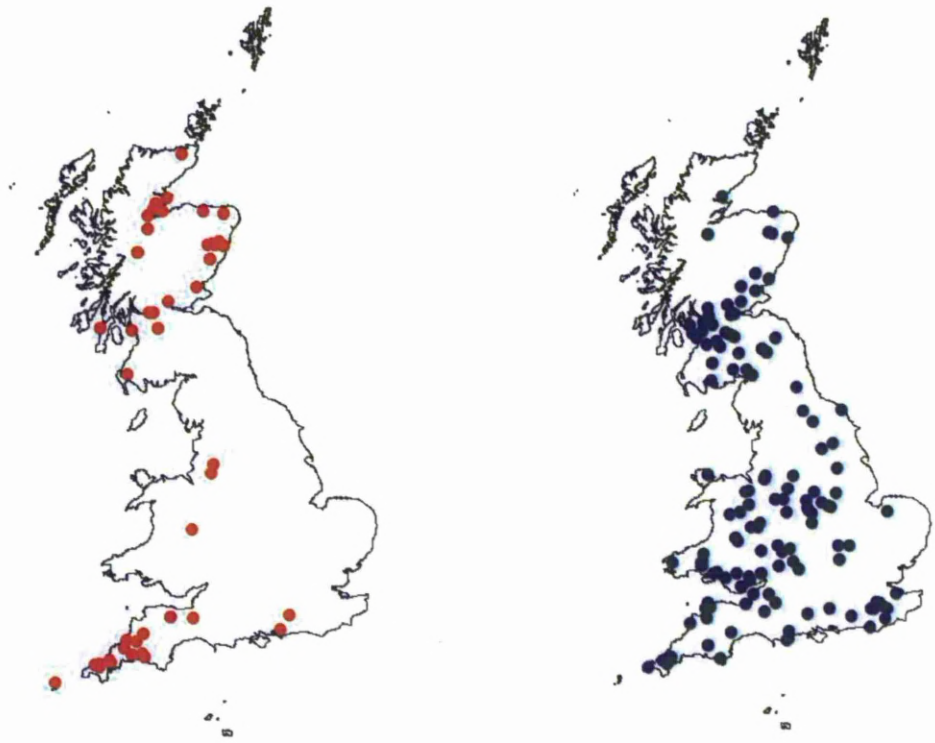
(a) September 2005 – March 2006

(b) September 2006 – March 2007

(c) September 2007 – March 2008

Figure 4.9: Geographical distribution of salmonellosis cases in all species (1st April 2005 – 31st March 2008) due to *S.* Typhimurium (a) DT40 and (b) DT56(v).

(a) DT40 (b) DT56(v)



Salmonellosis was suspected in 2 house sparrows (2 sites, 1 with DT40 and 1 with DT56(v)): the disease was confirmed in a second house sparrow submitted from each of these sites. Salmonellosis was also suspected in a single chaffinch (1 site with DT56(v)). All suspected salmonellosis cases occurred from November to February inclusive.

Nine birds, from separate sites, were suspected *Salmonella* sp. carriers, including 3 greenfinches (2 with DT40 and 1 with DT56(v)), 2 chaffinches (both DT40), a blue tit (DT120), and a siskin, a wood pigeon and a blackbird (each with DT56(v)). These cases were all examined from October to March inclusive. Four sites with suspected carrier birds had contemporaneous salmonellosis confirmed on PME in a greenfinch or house sparrow, including the site with a suspected carrier wood pigeon; a fourth site had multiple greenfinch mortality indicating that concurrent mortality due to salmonellosis

may have occurred. These findings indicate that these suspected carrier cases may have been birds in very early stages of clinical infection or asymptomatic carriers. The remaining 5 sites with suspected carrier birds, including the blackbird and blue tit, had no evidence of concurrent morbidity or mortality in other garden bird species.

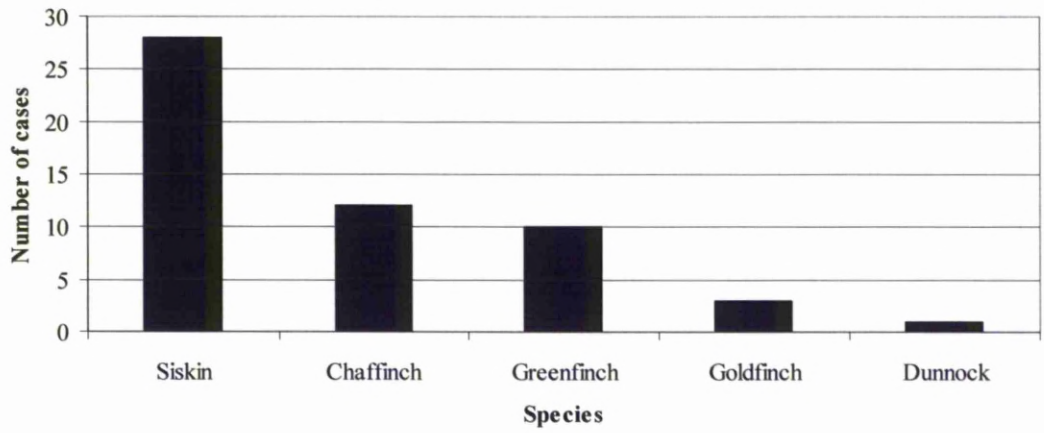
It was not possible to conclude whether 3 birds (2 with DT40 and 1 with DT56(v)) should be considered suspected salmonellosis or carrier birds, owing to the state of carcass preservation or information available, comprising a collared dove, siskin and chaffinch. Salmonellosis was confirmed in a goldfinch that died at the site from which the collared dove was submitted.

Salmonella arizonae was isolated from the intestinal tract of a juvenile blackbird examined in August 2005 that died as a result of trauma with concurrent severe endoparasitism; *S. arizonae* was considered most likely to be an incidental finding in this case.

Colibacillosis – *E. coli* serotype O86

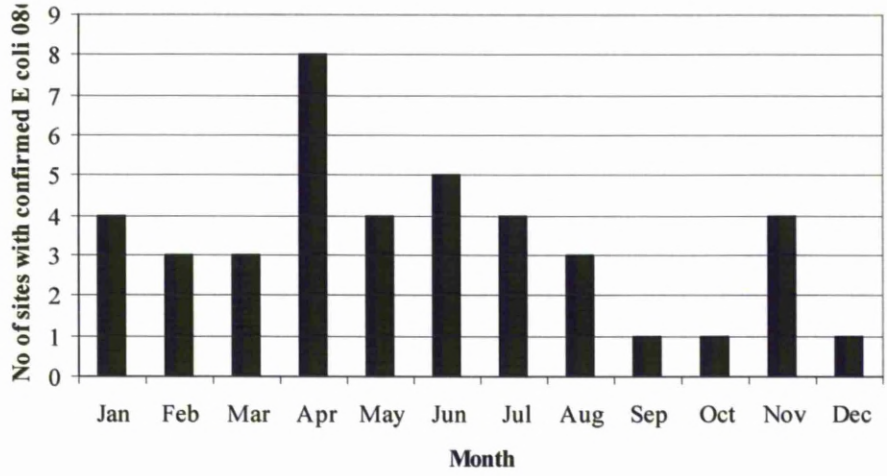
Bacteriological examination isolated non-lactose fermenting or late lactose fermenting *E. coli* isolates from 54 birds with API 20E profiles characteristic of *E coli* O86 (4144102 and 5144102), including 4 Fringillidae species (chaffinch, greenfinch, goldfinch, siskin) and 1 Prunellidae species (dunnock); of these 50 cases were confirmed as *E. coli* O86 by serology and the remaining 4 were not typed further. The siskin, chaffinch and greenfinch were the species in which the infection was most commonly diagnosed accounting for 52% (28/54), 22% (10/54) and 19% (12/54) of cases respectively (Figure 4.10).

Figure 4.10: Species breakdown of *E. coli* O86 infection (API 20E 4144102 and 5144102) (1st April 2005 – 31st March 2008).



These cases of *E. coli* O86 infection occurred across the calendar year with no clear seasonality, although the modal month for submissions was April (Figure 4.11).

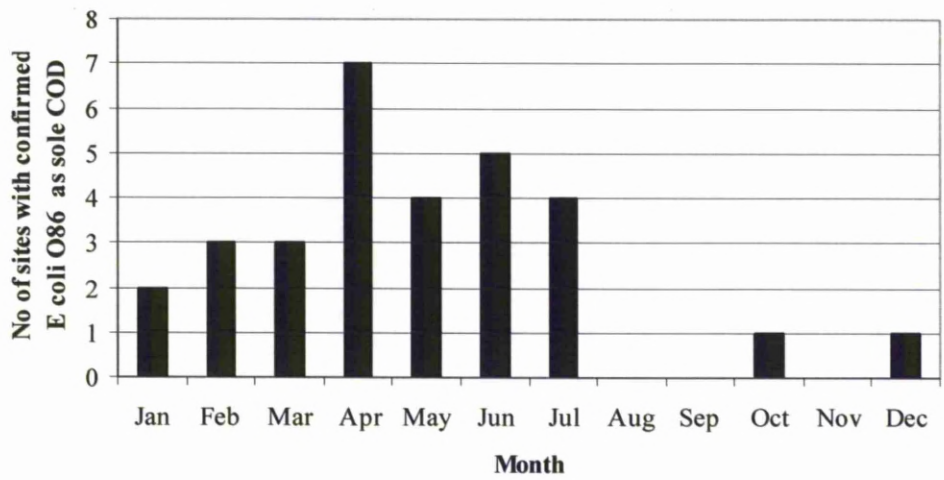
Figure 4.11: Total number of sites from which birds with *E. coli* O86 infection was confirmed by month (1st April 2005 – 31st March 2008).



E. coli O86 infection was considered to be the principal COD, and sole pathogen, for 43 of the 54 birds (Figure 4.12). Significant concurrent infectious disease was present in the remaining 11 birds: suspected or confirmed trichomonosis was present in 9 birds; trichomonosis and salmonellosis were present in a single greenfinch; and a single other greenfinch also had tick-related syndrome (see below).

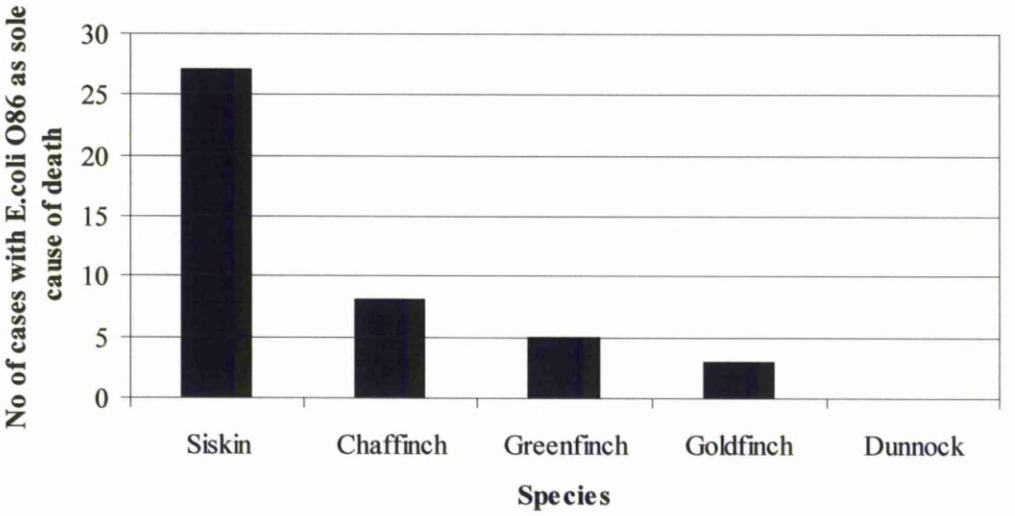
Sixty-six percent (20/30 sites) of the incidents where *E. coli* O86 was considered the primary and sole pathogen occurred between April and July: this is significantly greater than the number of incidents that would be predicted within this period if *E. coli* O86 disease events occurred evenly across the calendar year (X^2=5.40, df=1, P=0.02) indicating mortality incidents due to *E. coli* O86 infection may indeed be seasonal (Figure 4.12).

Figure 4.12: Total number of sites from which *E. coli* O86 infection in garden birds was confirmed (as the sole COD) by month (1st April 2005 – 31st March 2008).



The number of sites with confirmed *E. coli* O86 mortality incidents was relatively stable across the study period: 11 sites from 1st April 2005 to 31st March 2006, 9 sites from 1st April 2005 to 31st March 2006 and 7 sites from 1st April 2005 to 31st March 2006.

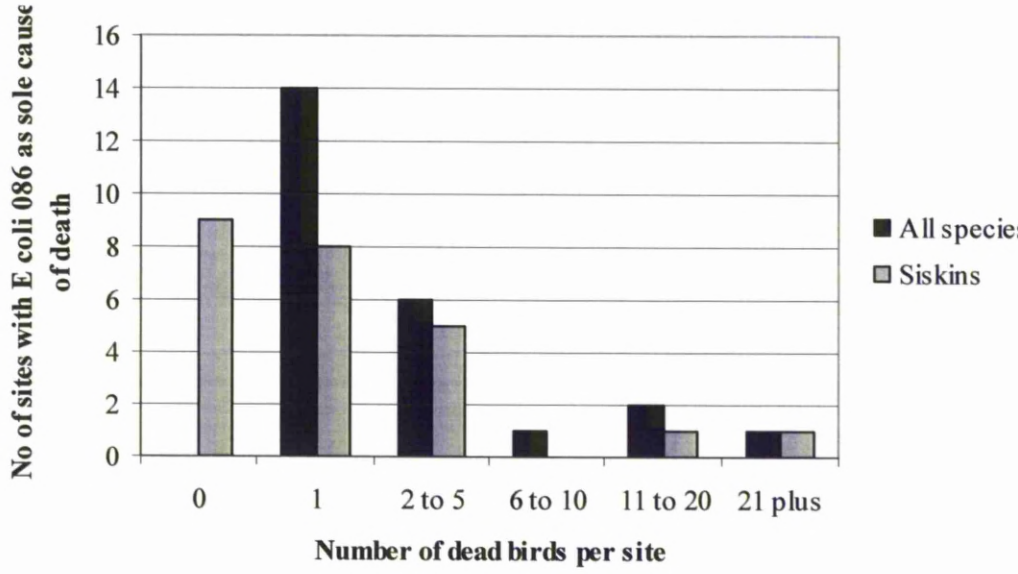
Figure 4.13: Species breakdown of cases where *E. coli* O86 infection (API 20E 4144102 and 5144102) was the sole COD (1st April 2005 – 31st March 2008).



Siskins comprised 63% (27/43) of the cases where *E. coli* O86 infection was the sole COD (Figure 4.13); *E. coli* O86 infection was the sole COD in all but 1 of the infected siskins which had concurrent trichomonosis. Siskin mortality due to *E. coli* O86 infection was confirmed at 48% (16/33) of sites where the infection was confirmed, and at 63% (15/24) of sites where infection was confirmed in bird(s) as the sole COD. Overall *E. coli* O86 infection was confirmed in a third (28/86) of the siskins examined at post mortem.

For sites where *E. coli* O86 infection was the sole COD, the total number of dead birds reported ranged from 1-26 (mean 3.69 +/- SEM 1.09) whilst the number of dead siskins ranged from 1-25 (mean 2.30 +/- SEM 1.02). Nine sites reported no siskin mortality (Figure 4.14).

Figure 4.14: Number of dead birds reported at each site with confirmed colibacillosis due to *E. coli* O86 infection as the sole COD in 1 or more birds at PME (1st April 2005 – 31st March 2008).



Birds with *E. coli* O86 infection were confirmed at sites from across Great Britain although the greatest number were from Scotland (Figure 4.15). *E. coli* O86 infection was considered to be the sole COD in birds submitted from sites north of a line between the estuaries of the Firth of Clyde to Firth of Forth in Scotland: the majority of these were in siskins. Breeding Bird Survey (BBS) data (Raven et al., 2004) illustrates that the distribution of *E. coli* O86 infection cases in siskins in Scotland matches their regions of greatest population density (Figure 4.16).

Figure 4.15: Distribution of sites from which *E. coli* O86 infection was confirmed (1st April 2005 – 31st March 2008).

Sites where *E. coli* O86 was considered the sole COD marked as a red circle.

Sites where concurrent disease was present marked in black circles.

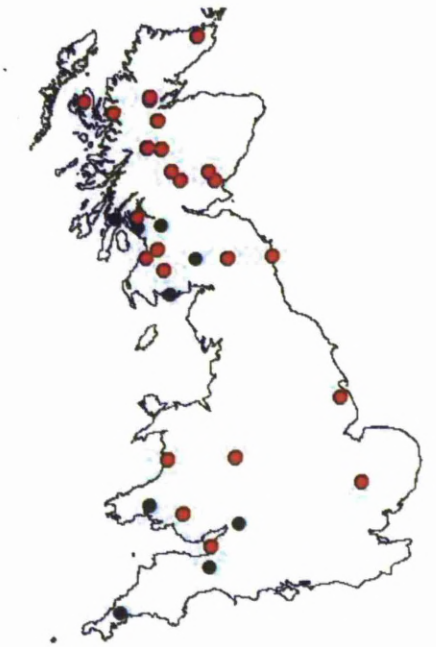
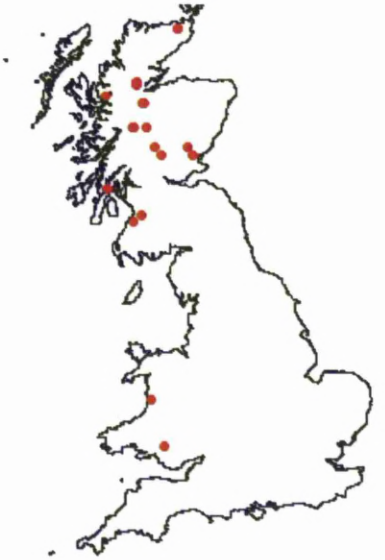


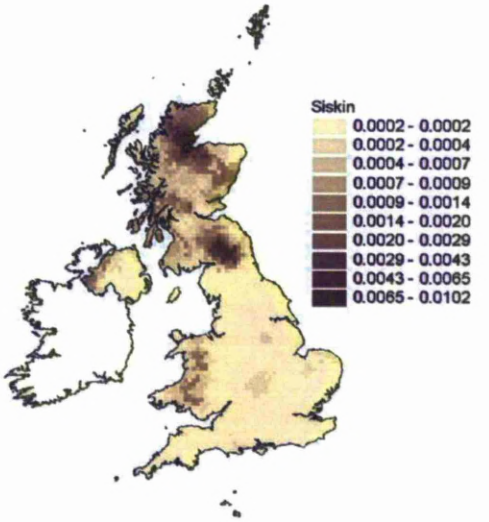
Figure 4.16: (a) Distribution of sites from which *E. coli* O86 infection was confirmed by species (1st April 2005 – 31st March 2008). (b) Population distribution data from the 2003 BBS survey (Raven et al., 2004) for each species. Graduated scale represents relative measure of abundance.

(a) *E. coli* O86 infection

(b) Species distribution

Siskin

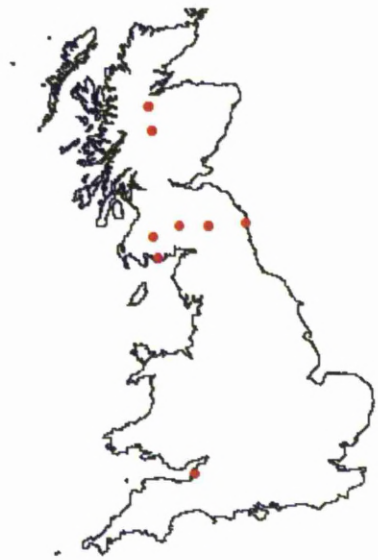


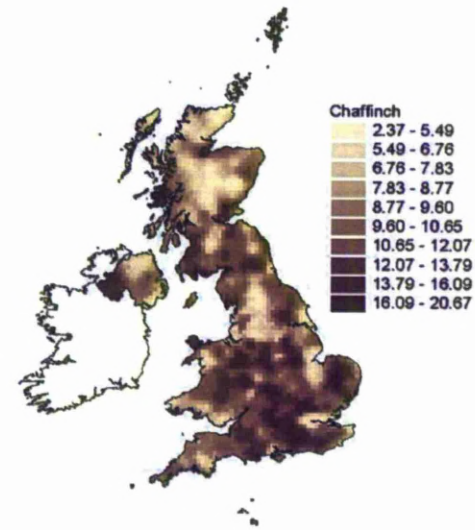


(a) *E. coli* O86 infection

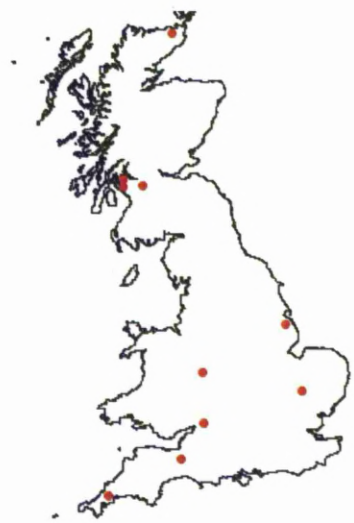
(b) Species distribution

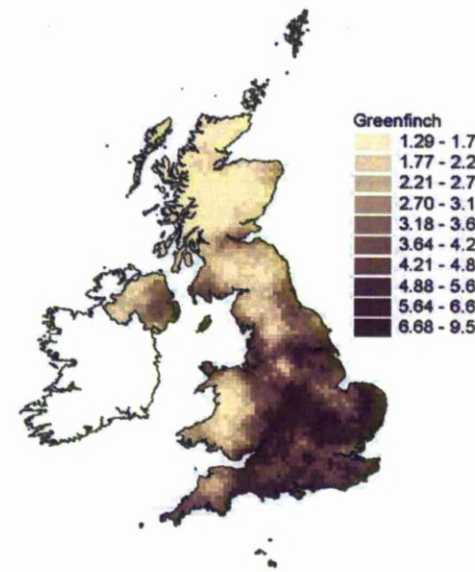
Chaffinch





Greenfinch





Of the 43 cases where *E. coli* O86 infection was considered the sole COD, liver culture was performed in 41 cases, of which all but 1 were positive for the bacterium. Similarly, small intestinal contents were cultured from all but 1 case and *E. coli* O86 isolated in every case, typically in confluent pure or nearly pure isolate. No other sites were

sampled for microbiological examination in these birds. Forty percent (17/43) of cases were in thin or emaciated body condition and the remaining 60% (26/43) were in normal condition. For those birds where the sex was identified, 80% (28/35) were male and 20% (7/35) were female. There were significantly more males than females confirmed with *E. coli* O86 infection as compared with a hypothetical bird population with a sex ratio of unity (X^2=11.429, df =1, P<0.001). This skew towards males was also present when siskins only were considered; 82% (18/22) were male and 18% (4/22) were female. There were significantly more male than female siskins confirmed with *E. coli* O86 infection as compared with a sex ratio of unity (X^2=7.682, df=1, P=0.006). 88% (37/43) of birds were adult, the remainder comprising 5 juveniles and a single nestling.

A description of the crop contents was available for 39 of the cases where *E. coli* O86 infection was considered to be the sole COD. The crop was empty in 62% (24/39) of birds; 38% (15/39) of birds had some food in the crop, and in 6 birds this was a large volume. A description of the gizzard contents was available for 38 of the cases where *E. coli* O86 infection was considered the sole COD. The gizzard was empty in 47% (18/38) of birds and the remaining 53% (20/38) of birds had some gizzard contents. Description of the small intestinal contents was available for 40 cases; 22 cases had contents that were described as autolysed and 9 birds had normal contents. Dark discolouration of the intestinal contents was present in 9 birds and the contents were cream/ yellow-coloured in 2 cases. The volume and consistency of intestinal contents was not fully described in every case; 2 birds had scant contents, a single bird had watery contents and a single bird had paste-like contents. Histopathology was not performed as autolysis precluded meaningful examination.

Non-lactose fermenting or late lactose fermenting *E. coli* isolates were also submitted for serology from 31 birds of 9 species which were seronegative for *E. coli* O86. The API 20E profiles for these isolates were non-characteristic for *E. coli* O86 in 27 cases (Table 4.3).

Table 4.3: Non-lactose fermenting or late lactose fermenting *E coli* isolates that were seronegative for *E. coli* O86.

API 20E profile	Number of birds	Species	Number of sites
4044502	5	3 greenfinch, 1 chaffinch, 1 bullfinch	5
4104102	1	1 robin	1
4140500	1	1 house sparrow	1
4144500	3	1 house sparrow, 1 goldfinch, 1 blackbird	3
4144502	3	1 house sparrow, 2 blackbird	3
5044502	3	3 greenfinch	3
5044552	2	2 greenfinch	2
5140500	1	1 house sparrow	1
5144502	6	3 house sparrow, 2 greenfinch, 1 marsh tit	4
5340500	1	1 greenfinch	1
6044503	1	1 greenfinch	1

Four birds with API 20E profiles typical of *E. coli* O86, but which were confirmed as seronegative, included a house sparrow and chaffinch (5144102), and a blackbird and jackdaw (4144102), each from separate sites.

Pasteurellosis

Pasteurellosis caused by *Pasteurella multocida* infection was confirmed in 14 birds (13 sites) of 9 species from a range of families including Columbidae (n=1), Fringillidae (n=3), Paridae (n=1), Passeridae (n=3), Prunellidae (n=2) and Turdidae (n=4). Cases were submitted throughout the calendar year and from across Great Britain with no evidence of temporal or spatial clustering (Figure 4.17). Sporadic mortality of individual birds was reported from each affected site, rather than multiple mortality events.

Figure 4.17: Geographical distribution of *Pasteurella multocida* cases (1st April 2005 – 31st March 2008).

P. multocida was isolated from the liver in 13/14 cases and from the small intestinal contents in 7/14 cases. Traumatic injuries characteristic of cat predation were present in 9 cases. *P. multocida* was cultured direct from a puncture wound in 2 cases (a robin and blackbird). 'Other trauma' of unresolved aetiology was present in 2 cases and no evidence of injury was present in the remaining 3 cases. No gross lesions thought to be associated with pasteurellosis were observed and *Pasteurella* sp. septicaemia was assumed in these cases. Affected birds included a range of age groups, comprising 1 nestling, 2 juvenile birds and 11 adults.

Unidentified *Pasteurella* spp. (on the basis of API profile) were isolated from 3 greenfinches and 2 collared doves submitted from 5 sites, all of which had suspected or confirmed concurrent trichomonosis. The *Pasteurella* spp. were isolated from necrotic ingluvitis lesions in 3 cases, and from the liver in 2 cases, and were assumed to have acted as secondary or opportunist pathogens in these birds.

Yersiniosis

Yersiniosis, caused by *Yersinia pseudotuberculosis*, was confirmed in 8 birds (8 incidents) from 3 finch species (3 chaffinch, 2 goldfinch, 1 hawfinch), a barn swallow and a blackcap. Gross lesions associated with *Y. pseudotuberculosis* infection were present in all birds, characterized by multiple focal necrotic lesions in organs including the liver, spleen, lung, focal lesions in the appendicular skeletal system including joints, muscle and bone, and diffuse lesions with fibrinous polyserositis. Crush-preparations from extravisceral lesions, from sites including the shoulder joint, temporal region and tibiotarsus of affected birds, were negative for acid-fast organisms on Ziehl-Neelsen staining.

The COD was considered to be solely infectious disease in 7 cases; *Y. pseudotuberculosis* was considered to contribute significantly to the COD in all 8 cases. Significant concurrent infectious disease was confirmed in 2 cases; focal lesions due to *S.* Typhimurium DT40 infection in a chaffinch and necrotic ingluvitis due to trichomonosis in a chaffinch. Protozoal oocysts (species unidentified) were present in the intestinal contents of a goldfinch and the blackcap that may have adversely affected the birds' health. The blackcap also had concurrent parasitic infection with adult air sac nematodes, *Diplotriaena tridens*, and intestinal infection with a *Campylobacter* sp.; these findings might have contributed to ill health or have been incidental.

Affected birds were adult with no evidence of gender bias (4 male, 4 female) and were in poor body condition (thin or emaciated) in all cases with one exception, the blackcap,

where the COD was considered to be a combination of infectious disease and trauma. This bird had a fractured coracoid suggesting that collision may have been the ultimate COD; the bird may have been in a weakened state due to the bacterial and concurrent parasitic infections, predisposing to trauma.

Single birds were reported to be affected in 5 of the incidents. Multiple birds of the same species were reported to be affected in 3 incidents, however, these included the 2 incidents where significant concurrent infectious disease frequently associated with multiple mortality incidents, salmonellosis and trichomonosis, was diagnosed.

Yersiniosis cases showed strong seasonality during the colder months of the year with 7/8 cases confirmed from January to April inclusive. The cases occurred sporadically over the 3-year study period (4 sites from 1st April 2005 to 31st March 2006, 3 sites from 1st April 2006 to 31st March 2007 and 1 site from 1st April 2007 to 31st March 2008). Incidents occurred in central southern and south-western England, Wales and Scotland (Figure 4.18).

Figure 4.18: Geographical distribution of Yersiniosis cases (1st April 2005 – 31st March 2008).

***Suttonella ornithocola* infection**

Suttonella ornithocola infection was diagnosed in a total of 4 blue tits (4 sites) over the study period. *S. ornithocola* was isolated from the lung of all 4 birds and from the liver in 1 of the birds affected. All *S. ornithocola* isolates had morphological and culture characteristics typical of those reported previously for this bacterium (Foster et al., 2005; Kirkwood et al., 2006). For each isolate, 16S rRNA sequence data was identical to that of the type strain (Genbank Accession Number AJ717394). Tit species were submitted from mortality incidents throughout Great Britain and *S. ornithocola* infection was confirmed in England and Wales (Figure 4.19).

Figure 4.19: Distribution of the mortality incidents from which tit carcasses were examined during the study period (1st April 2005 to 31st March 2008). Red circles indicate the distribution of mortality incidents from which *Suttonella ornithocola* was isolated. Black dots indicate the distribution of other mortality incidents from which tit carcasses were examined.

Details of *S. ornithocola*-positive mortality incidents are given in Table 4.4. Several individuals of multiple tit species were seen to be affected in 3 of the 4 incidents. Mortality or morbidity of blue tits was reported in each incident. Where sick birds were observed, they exhibited non-specific signs of malaise, such as lethargy and fluffed-up plumage. No evidence of contemporaneous morbidity or mortality was observed in other garden bird species at affected sites.

Table 4.4: Details of mortality incidents from which birds were submitted and *Suttonella ornithocola* was isolated (1st April 2005 to 31st March 2008). The total number of birds found dead per incident is given, with the total number of birds seen sick per incident in parentheses.

Incident number	Species affected Number found dead (seen sick)	Month and Year	County	Clinical signs
Incident 1.	Blue tit 6(0) Coal tit 0 (1)	April 2005	Cambridgeshire, England	Lethargic
Incident 2.	Blue tit 1(3) Great tit 1(0)	April 2006	Kent, England	Fluffed up and lethargic
Incident 3.	Blue tit 1(1) Coal tit 0(1) Long-tailed tit 0(1)	April 2007	Monmouthshire, Wales	Fluffed up
Incident 4.	Blue tit 1(1)	April 2007	Essex, England	Fluffed up and lethargic

A summary of the details of the confirmed cases of *S. ornithocola* infection is presented in Table 4.5. Typically, poor facial plumage condition was observed with peri-oral matting of feathers, and there usually was urate staining around the vent. In each case, the upper alimentary tract was empty and the gizzard contained only dark-stained grit, indicating that the bird had not recently fed. Pulmonary congestion was noted in 2 cases, whilst the respiratory tract appeared grossly normal in the remaining 2 birds. No other significant abnormalities were noted. All cases were male adult birds.

Histopathological examination was performed on the lung and other tissues from 2 of the birds from which *S. ornithocola* was cultured (Table 4.5). In 1 of the birds there was acute necrotising pneumonitis with multiple foci of pulmonary necrosis associated with clusters of Gram-negative rods (Incident 1) (Figure 4.20). A mixture of Gram-positive and Gram-negative rods was present in some of the air spaces and surrounding tissue in the other bird (Incident 2), but there was no evidence of tissue necrosis. The lung of a single bird (Incident 2) was markedly congested and a small number of parabronchi contained acellular eosinophilic material (oedema fluid). Large mononuclear cells with foamy cytoplasm (macrophages) and lymphocytes were present within, and lining, the air spaces in this bird (Incident 2). It is possible that some of the foamy cells lining the air spaces were reactive type II pneumocytes, but no additional investigations were

conducted to investigate this further. No abnormalities were detected in the other organs examined.

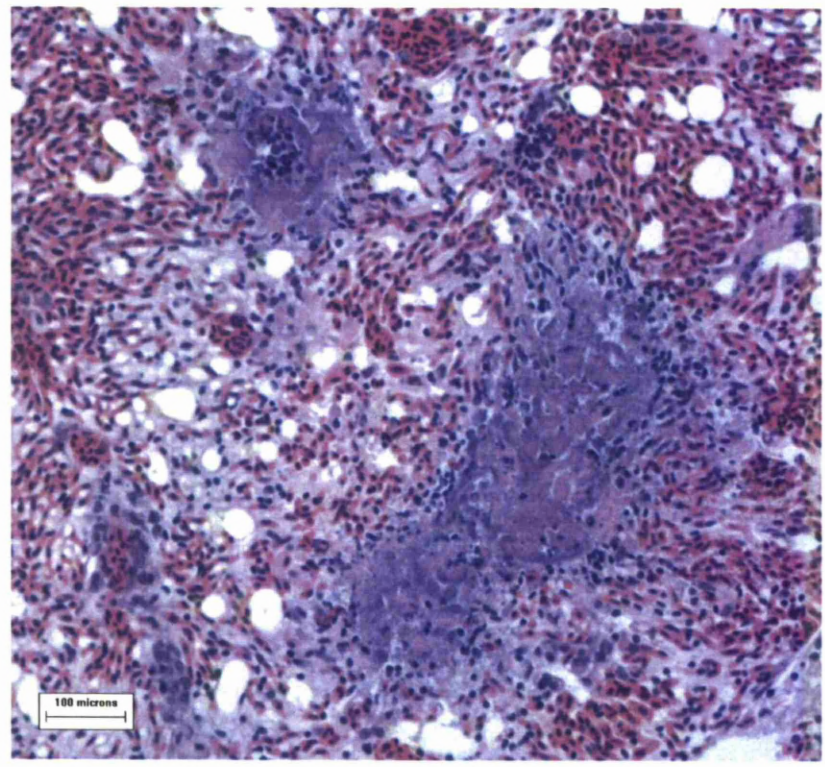
Table 4.5: Summary of the tit cases from which *Suttonella ornithocola* was isolated (1st April 2005 – 31st March 2008).

Incident Reference number	Species	Age	Sex	Body weight (g)	Body condition	Lung congestion present?	Tissues cultured for bacteria	State of carcass preservation	Histo-pathology
Incident 1.	Blue tit	Adult	Male	9.9	Good	No	Heart blood, liver, small intestine (SI), lung*	Mild autolysis	Heart, kidney liver, lung
Incident 2.	Blue tit	Adult	Male	7.9	Thin	No	Liver*, SI, lung*	Moderate decomposition	Brain, heart, kidney, liver, lung, pectoral muscle, trachea
Incident 3.	Blue tit	Adult	Male	8.3	Thin	Yes	Liver, SI, lung*	Moderate decomposition	NA
Incident 4.	Blue tit	Adult	Male	8.0	Thin	Yes	SI, lung*	Moderate decomposition	NA

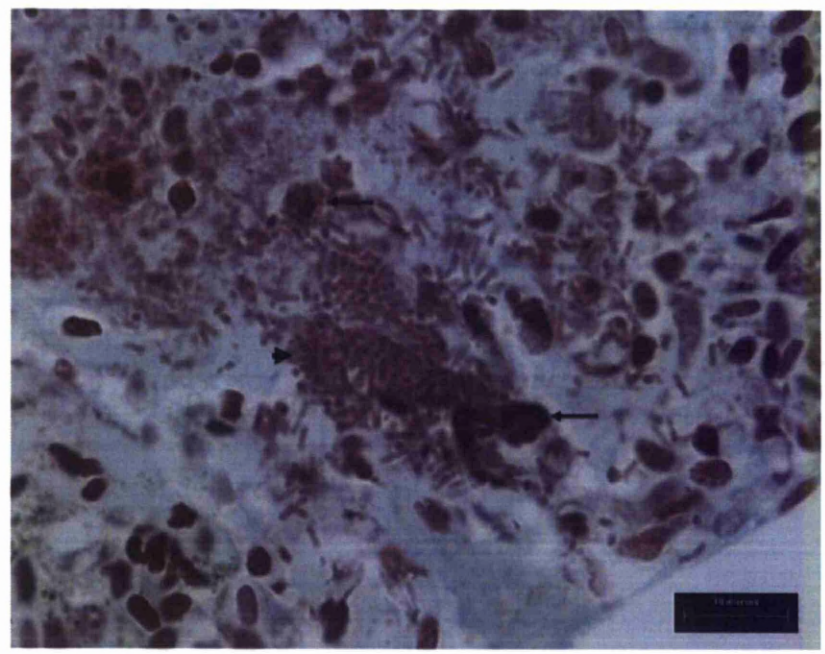
*Positive for *Suttonella ornithocola*

Figure 4.20: Acute necrotising bacterial pneumonitis in a blue tit (Incident 1) (a) Haematoxylin and Eosin (b) Gram-Twort. Note colony of Gram-negative rods (arrowhead) and small number of mononuclear inflammatory cells (arrows).

(a)



(b)



Avian tuberculosis

Avian tuberculosis was confirmed in a single adult male wood pigeon submitted from Kent in December 2007. No sign of concurrent morbidity or mortality affecting other species had been observed at that site. The bird had splenomegaly, hepatomegaly, fibrinous pericarditis and perihepatitis on gross examination and was emaciated (Figure 4.21). Mild stress marks were present in the tail feathers and there was partial depigmentation of primary 5. on the left wing; no other plumage abnormalities were noted. Numerous acid-fast rods were visible on Ziehl-Neelsen staining of hepatic and splenic lesions. No mycobacterial culture for speciation was performed.

Figure 4.21: Fibrinous pericarditis and perihepatitis and hepatomegaly in a wood pigeon with avian tuberculosis.

FUNGAL

Aspergillosis (*Aspergillus fumigatus* and *Aspergillus* spp.) and 'unidentified' fungal infection

Aspergillosis was diagnosed in 6 birds (6 sites) from 4 species (blackbird (n=3), great spotted woodpecker (n=1), house sparrow (n=1) and wood pigeon (n=1)) where gross or

histopathological lesions consistent with fungal infection were recorded in combination with isolation of an *Aspergillus* sp. on lesion culture. Respiratory tract lesions were grossly visible in all but 1 of these birds, including focal (single or multiple) lesions in the lung of 4 birds, and single birds with an air sac plaque or tracheal lesion. 'Unidentified' fungal infection was diagnosed on the basis of histological or cytological examination in a further 5 birds (5 sites) of 3 species (blackbird (n=3), blue tit (n=1) and dunnock (n=1)). Microbiological examination of the lesion(s) was not performed in 4 of these birds therefore the fungal pathogen(s) could not be identified.

The fungal pathogen(s) was considered to be a significant contributory factor to the COD for all the aspergillosis and 'unidentified' fungal infection cases. Two of these birds were euthanased on welfare grounds and a single bird died as a result of trauma.

The blackbird is the species in which aspergillosis and other unidentified fungal infections were most frequently diagnosed, accounting for over half of these cases (55% - 6/11 birds). Single blackbirds were submitted from each site. Single cases were confirmed in the other affected species including the blue tit, dunnock, great spotted woodpecker, house sparrow and wood pigeon.

Juvenile birds accounted for 36% (4/11) of the cases of aspergillosis and fungal infection and these were submitted between June to July inclusive. Three juvenile blackbirds with aspergillosis or respiratory tract fungal infections had concurrent mixed endoparasitic burdens, and poor body condition, indicating a general state of immunosuppression and debility. A single great spotted woodpecker with mixed parasitic and fungal infection was submitted to a rehabilitation centre before being euthanased on welfare grounds.

A single wood pigeon had a distal tracheal lesion obstructing the lumen (Figure 4.22). The bird was otherwise in good body condition and examination of the digestive tract indicated that it had been feeding well. The bird was observed to fall from the sky to its death during flight indicating peracute death consistent with asphyxiation.

Figure 4.22: Aspergilloma within the tracheal lumen of a wood pigeon.

Fungal necrotising orchitis and tubulonephritis was diagnosed in a first year blue tit.
An adult female blackbird in normal body condition was euthanased on welfare grounds due to severe fungal dermatitis with lesions affecting the head, body and wings from which a *Penicillium* sp. and *Mucor* sp. were isolated in combination with a *Staphylococcus*. sp.

Fungal infection cases were submitted across the 3-year study period from counties in England, Wales and Scotland with no evidence of spatial clustering.

Aspergillus sp. were isolated from upper alimentary tract lesions from a further 4 birds (4 sites), comprising 3 greenfinches and a bullfinch, all of which died as a result of concurrent trichomonosis. The fungal isolates were considered likely to be secondary opportunistic infections in these birds.

PARASITIC

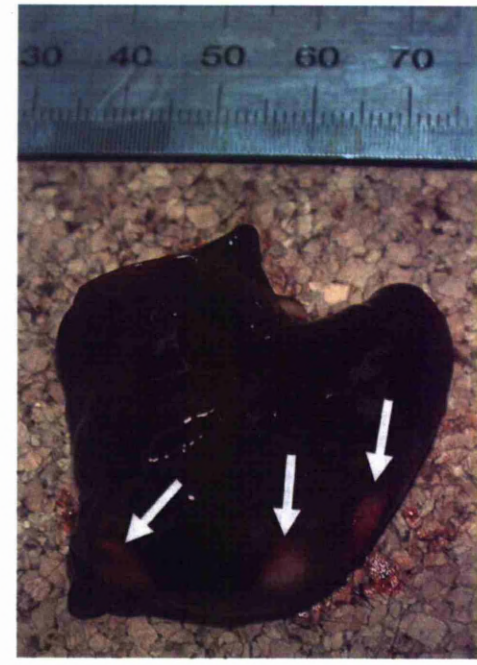
Trichomonosis

Trichomonosis caused by *Trichomonas gallinae* was diagnosed in a total of 629 birds (531 confirmed and 98 suspected) of 17 species from 492 sites over the study period. Necrotic pharyngitis was typically observed in trichomonosis cases in columbiform species (Figure 4.23a). Extra-alimentary lesions were present in the liver of a single collared dove with trichomonosis (Figure 4.23b): motile trichomonads were cultured from both the hepatic and oropharyngeal lesions. Necrotic ingluvitis was seen in the passeriform species affected (Chapter 6, Figure 6.3).

Figure 4.23: Gross lesions of trichomonosis in columbiform species (a) Caseous oropharyngeal lesion (Black arrow) (b) Focal hepatic lesions caused by trichomonosis in a collared dove (White arrows).

(a)

(b)



Finch trichomonosis was diagnosed as an emerging infectious disease of British finches in 2005 (Holmes et al., 2005; Pennycott et al., 2005). The epidemiology of

trichomonosis in British garden birds, particularly Fringillidae species, is investigated in detail in Chapters 6-9.

Coccidiosis

Coccidiosis was considered a significant contributory factor to the COD in 30 birds with moderate to numerous protozoal oocysts present within the small intestinal contents. These comprised 24 finches (greenfinches (n=22), chaffinch (n=1), goldfinch (n=1)), 4 sparrows (house sparrow (n=2), tree sparrow (n=2)) and 2 dunnocks. These were all submitted from separate sites except the tree sparrows which were submitted from the same garden. Juveniles accounted for 40% (12/30) of cases with the remainder classed as adult. There was no evidence of a gender skew (12 male, 8 female, 10 undetermined).

Significant concurrent infectious disease was observed in the majority of cases: 21 birds had concurrent trichomonosis, 1 had salmonellosis and 1 had a concurrent *E. coli* O86 infection and trichomonosis. Coccidiosis was considered the primary pathogen in 5 birds. Both affected tree sparrows ultimately died as a result of predation. The majority of coccidiosis cases were found from July to October (77% - 23/30 birds): this reflects the seasonality of trichomonosis commonly seen in these birds. Birds with coccidiosis were submitted with wide geographical distribution across Great Britain.

Protozoal oocyst species identification was not performed on the majority of these cases. Samples from a small number of birds were examined by Professor S.J. Ball who tentatively identified the following species on the basis of their oocyst morphology and dimensions: *Isospora chloridis* in the greenfinch, *Isospora fringillae* in the chaffinch and *Isospora lacazei* in the house sparrow.

Helminthosis

Helminthosis was considered to be significant to the COD in 4 juvenile blackbirds and to 2 adult starlings submitted from separate sites from across Great Britain. A combination of nematode (*Capillaria* spp., *Syngamus trachea*), cestode (*Porrocaecum* spp.) and acanthocephalan parasites were present. Numerous gross parasites were visible

in these birds. Parasite species identification was not performed in these cases. Five of the birds were in poor body condition (4 thin and 1 emaciated): a starling with syngamiasis and no other gross parasites was in normal body condition.

Gross parasites were frequently present in Turdidae and Sturnidae species: in addition, examination of small intestinal contents identified nematode and cestode ova in a number of birds of these species. In total, 76% (67/ 88) of blackbirds had evidence of nematode or cestode parasites, comprising 40 in the gastro-intestinal (GIT) tract alone, 15 with GIT parasites and syngamiasis and 2 with syngamiasis alone. In total, 29% (9/31) of starlings had evidence of nematode or cestode parasites, comprising 4 in the GIT tract alone, 2 with GIT parasites and syngamiasis and 3 with syngamiasis alone. Whilst these parasitic infections were not considered significant to the COD, and most likely to represent an incidental finding in the majority of cases, it is possible that helminthiasis may have compromised these birds to some extent.

VIRAL

Avian poxvirus infection

Avian pox was confirmed in 7 birds and suspected in 8 birds submitted from 13 sites. These comprised 7 great tits (6 sites), 4 dunnocks (4 sites), 3 house sparrows (2 sites) and 1 wood pigeon (1 site) (Table 4.6).

Table 4.6: Summary of the avian pox cases (1st April 2005 – 31st March 2008).

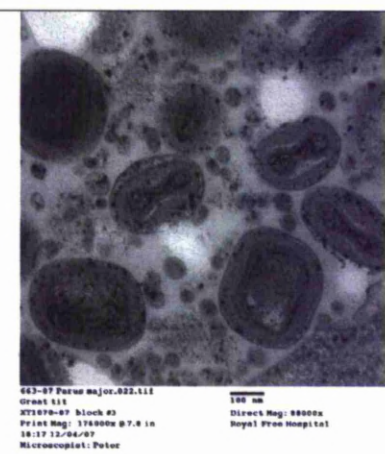
Case number	Species	Age	Sex	Body condition	Lesion	Suspected or confirmed diagnosis	Wet or dry pox	Significant or incidental to the COD	Month – Year
1	Dunnock	Adult	Female	Thin	Head	Suspected	Dry	Significant	Aug-06
2	Dunnock	Juvenile	Undeter-mined	Thin	Head	Suspected	Dry	Significant	Aug-07
3	Dunnock	Juvenile	Undeter-mined	Thin	Legs and head	Confirmed histo	Dry	Significant	Sept-07
4	Dunnock	Adult	Female	Thin	Feet	Suspected	Dry	Incidental	Oct-07
5	Great tit	Adult	Male	Thin	Head	Suspected	Wet	Significant	Mar-07
6	Great tit	Juvenile	Undeter-mined	Normal	Body	Suspected	Dry	Significant	Aug-07
7	Great tit	Undeter mined	Undeter-mined	Normal	Head and wings	Confirmed histo	Dry	Significant	July-07
8	Great tit	Adult	Undeter mined	Normal	Head and wings	Confirmed histo and EM	Dry	Significant	Sept-07
9	Great tit	Adult	Undeter-mined	Normal	Head	Confirmed histo	Dry	Significant	July-07
10	Great tit	Adult	Female	Normal	Head	Confirmed on EM	Dry	Significant	Sept-07
10	Great tit	Adult	Male	Normal	Head	Confirmed on EM	Dry	Significant	Sept-07
11	House sparrow	Adult	Male	Emaciated	Head	Suspected	Dry	Significant	Aug-05
12	House sparrow	Adult	Male	Thin	Head	Suspected	Dry	Significant	Oct-05
12	House sparrow	Adult	Female	Thin	Head	Suspected	Dry	Significant	Oct-05
13	Wood pigeon	Adult	Female	Thin	Head	Confirmed histo	Dry	Significant	Feb-06

Lesions were typically present on the head only (particularly affecting the periocular and perioral skin) (9 cases), infrequently on multiple areas of the body (4 birds), and less commonly on the feet only (1 case) (Figure 4.24). Cutaneous or 'dry pox' was the most common presentation (13 cases in 4 species). Concurrent oral lesions were present in 1 great tit and 1 wood pigeon, in combination with cutaneous lesions; these cases might have been 'wet' pox, however, histopathological examination of the extra-cutaneous

lesions to confirm their aetiology was not possible due to rapid autolysis of the alimentary mucosa. Bacteriological examination of skin lesions was performed in 8 cases and *Staphylococcus* spp. were isolated in 7 cases (2 beta-haemolytic *Staph.* sp., 2 *S. aureus* and 3 non-haemolytic *Staph.* sp.). Avian pox infection was considered to be a significant contributory factor to the COD for all cases except a single dunnock, with mild lesions restricted to the feet, which died as a result of suspected trichomonosis. Avian pox lesions may have impaired the birds' predator avoidance capabilities, through compromised vision or flight, and predation injuries were present in 5 birds. Splenomegaly, suggestive of terminal bacteraemia and septicaemia, was present in 2 birds. Concurrent infectious disease (trichomonosis) was considered to contribute to the ultimate COD in 2 birds and a third bird was euthanased on welfare grounds. Affected birds of non-Paridae species were typically in poor body condition (7 thin and 1 emaciated), contrasting with the findings in Paridae species (6 normal and 1 thin).

Cutaneous histopathology in avian pox cases confirmed severe epidermal ballooning hyperplasia, multifocal coalescing areas of degeneration and eosinophilic intracytoplasmic inclusion bodies characteristic of Bollinger bodies. Electron microscopy confirmed the presence of multiple virions with a characteristic size and profile for avian pox (Figure 4.24f).

Figure 4.24: Avian pox lesions (a) great tit at feeding station (b) moribund great tit (c) great tit at PME (1) (d) great tit at PME (2) (e) avian pox caseous lesion in transverse section (f) transmission electron micrograph of avian pox virions of characteristic size and profile.

(a) Credit to G. Morris	(b) Credit to D. Bakewell
	
(c)	(d)
	
(e)	(f)
	

Avian pox was confirmed across age classes (3 juvenile, 12 adult and 1 undetermined) and there was no evidence of a gender bias (5 female, 4 male, 7 undetermined).

Avian pox infection was seasonal, with the majority of birds (87% -14 birds, 12 sites) found between July and October. Avian pox incidents in non-Paridae species were confirmed in each year of study: 2 incidents in both 2005 and 2006 and 4 incidents in 2007. Avian pox was confirmed for the first time in Paridae species in 2007, in 6 incidents. The distribution of avian pox incidents in Paridae species was restricted to England, with the majority clustered in the south-eastern counties (Figure 4.25). Incidents occurred in non-Paridae species across Great Britain. The incidents were widely dispersed across years and the spatial distribution did not cluster with that of the novel incidents involving Paridae species.

Figure 4.25: Distribution of (a) Paridae and non-Paridae incidents of avian pox (b) Non-Paridae incidents of avian pox infection by year.

(a) Paridae incidents shown in green. Non-Paridae incidents shown in blue.

(b)Non-Paridae incidents in 2005 shown in turquoise, in 2006 shown in pink and in 2007 shown in orange.

Avian pox incidents involving non-Paridae species all included reports of a single species affected: 5 sites reported an individual bird observed sick (or dead) and 2 observed 2 birds affected. Avian pox incidents in Paridae species involved great tit only at 3 sites, great tit and blue tit at 2 sites and blue tit only at a single site. The number of birds seen affected ranged from 1-20 (mean=7.8) and the number of birds found dead ranged from 1-3 per incident. Individual birds could be told apart with high confidence due to variation in lesion distribution and severity. Most of the sites were garden feeding stations although the greatest number of affected birds was observed at a field study site.

OTHER

Drowning or hypothermia

Drowning, or hypothermia secondary to water-logging of plumage, was suspected to be the COD in 3 starlings submitted from a single site in northern England. All examined birds were juveniles with dull grey brown plumage and the incident occurred in May indicating that the birds were likely to be the first brood of the year. Mass mortality was reported with 28 dead birds found at the site on the same morning in and around a garden pond. The birds were in good body condition with no other abnormalities visible. Microbiological examination was unremarkable and tissues were negative for pesticide residues under the Wildlife Incident Investigation Scheme (HSE 2010).

Oesophageal obstruction

Complete obstruction of the distal oesophagus at the level of the furcula by a large piece of peanut was observed in 2 adult birds at different sites: a male robin in January 2006 and a dunnock of undetermined sex thought to be in its first year in July 2006 (Figure 4.26). Both birds were in thin body condition, had not eaten recently and had no other significant abnormalities on PME examination.

Figure 4.26: Complete obstruction of the oesophagus with a peanut fragment in (a) a dunnock – peanut *in situ* at the level of furcula and (b) a dunnock – upper digestive tract dissected and peanut exposed.

(a)

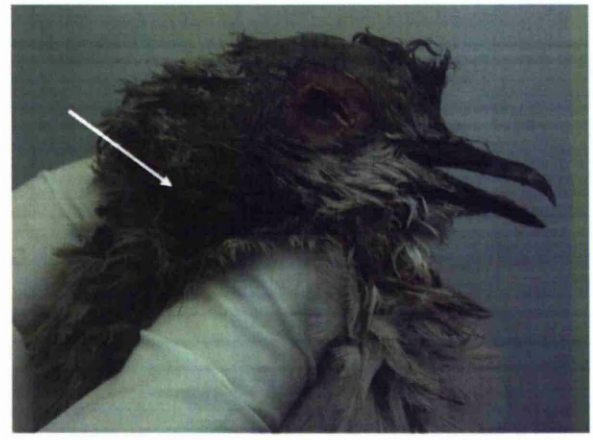
(b)

Tick-related syndrome

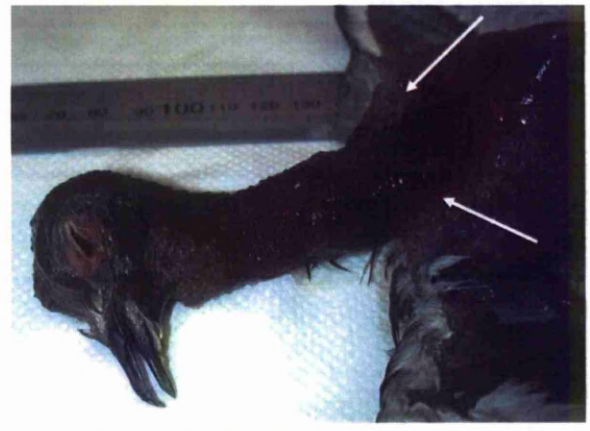
Tick-related syndrome (TRS) was diagnosed as the COD of 3 adult birds from 3 sites including a collared dove, house sparrow and a greenfinch. Engorged *Ixodid* sp. adult female ticks (1-4) were present on the head of affected birds. The HPA confirmed these to be *I. frontalis*. Peracute localized haemorrhage was present in the soft tissue adjacent to the tick attachment site (Figure 4.27). Birds were in good body condition and some had eaten recently, with a normal volume of ingesta in the gizzard.Cases were distributed across England in Cambridgeshire, Devon and Suffolk between August and October. TRS was suspected in a blackbird submitted from Hertfordshire in July based on the appearance of localized peracute haemorrhage: however, no tick or attachment site was visible.

Figure 4.27: (a) Two engorged *I. frontalis* ticks (white arrow) attached to the head of a collared dove. Note the periocular swelling and haemorrhage. (b) Severe swelling and subcutaneous haemorrhage of the head and neck: after removal of plumage. (c) after reflection of the skin with the coelomic cavity exposed. White arrows in (b) and (c) indicate sharp delimitation between the haemorrhagic and normal tissue.

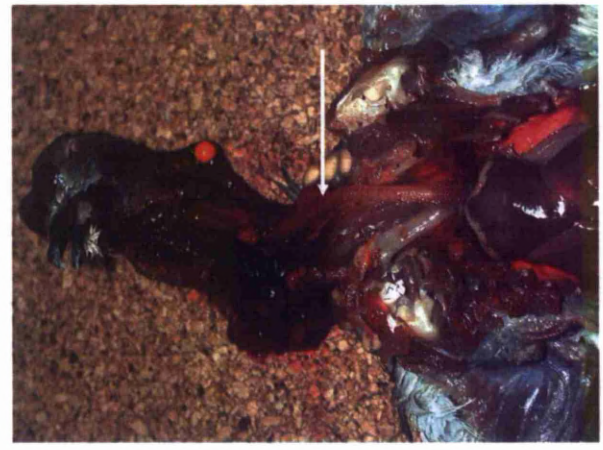
(a)



(b)



(c)



4.4 DISCUSSION

Marked variation was found in the importance of infectious disease as a COD between avian families in this study. Gregarious and principally granivorous species were most frequently affected by infectious disease, chiefly the finches, sparrows, pigeons and doves. Whether these species are intrinsically more susceptible to these conditions, or their behaviour increases the risk of pathogen exposure is unknown. Jennings (1961) noted that colonial nesting species were predisposed to disease outbreaks (e.g. Passeridae): it is likely that high rates of intraspecific contact in social species might facilitate pathogen transmission. Brittingham et al. (1986) found that the house sparrow and American goldfinch (*Carduelis tristis*) were the species most frequently reported in mortality incidents (with causes other than predation or window collision) in the U.S.A. and also noted the gregarious habit of these species during winter months at garden feeding stations, with communal roosting and flock feeding. As with this study, mortality of Paridae species was infrequently reported (Brittingham et al., 1986).

Salmonellosis

Salmonellosis was diagnosed most frequently during the winter months and in Fringillidae and Passeridae species in this study, a male bias was observed in greenfinch cases: these findings were similar to the study of passerine mortality due to salmonellosis across Britain 1993-2003 inclusive (Chapter 2). The geographical distribution of salmonellosis incidents corroborated the findings from 1993-2003 with a notable absence of incidents from East Anglia. This geographical trend was confirmed through the systematic surveillance scheme increasing confidence that it represents a true finding: potential explanations are discussed in Chapter 2.

Over this study period dramatic changes occurred, with a marked reduction in the overall number of salmonellosis incidents and shift in the major *S.* Typhimurium phage type from DT40 (1993-2003 - Chapter 2) to DT56(v) (2005-2008). The explanation for the decline in salmonellosis incidents is unclear. Since salmonellosis occurs chiefly

during the winter months, climatic trigger factors have been proposed where harsh winters or particularly inclement weather might increase disease incidents (Kirkwood et al., 1997; Daoust et al., 2000). The winters over this study period were mild, with positive mean temperature anomalies (as compared with the period 1971-2000) recorded for the UK each January (Met Office 2010). It is interesting that January 2007 (mean temperature anomaly +2.6 °C) was the warmest on record for the period 2001-2010 inclusive, indicating that mild winter weather might have contributed to a decline in salmonellosis incidents that year. January 2008, however, whilst mild (mean temperature anomaly +1.9 °C) was less so than the preceding year indicating that climate is unlikely to account for the marked year on year decline in salmonellosis incidents. Salmonellosis incidents recorded by the GBH*i* have continued to decline sharply to very low numbers in the winter of 2008/9 and 2009/10 (IoZ, SAC, *unpublished data*); importantly the winter months were cooler than average in these 2 years (UK January mean temperature anomaly for 2009 of -0.5 °C and 2010 of -2.5 °C). Collectively, these findings indicate that changes in winter climate are unlikely to be the sole or major explanation for the decline in salmonellosis incidents observed from 2005 to 2008. However, since the available climatic data is summarized by month and region it is not possible to investigate daily variation in local climatic conditions at the sites where disease incidents occurred.

Whilst salmonellosis was the most frequently diagnosed infectious disease of British garden birds over the period 1993-2003 (inclusive) (Chapter 2), the emergence of trichomonosis in 2005 and subsequent epidemic mortality led the protozoal disease to supplant this position. Greenfinch was the species most commonly affected by both salmonellosis and trichomonosis in this study. Significant decline in the breeding greenfinch population occurred following the trichomonosis epidemic of 2006 (Chapter 6) and there is evidence to support further decline following the 2007 epidemic (Chapters 7, 12) although the extent of this has yet to be quantified. The reduction in salmonellosis incidents occurred over this same 2-year period. The greenfinch population decline occurred chiefly in England in the region of high trichomonosis-associated mortality (Chapter 6). It is noteworthy that in the winter of 2007/8,

salmonellosis incidents were restricted chiefly to Scotland, out with the peak epidemic region for trichomonosis although the parasite had been confirmed across Great Britain. Given the temporal and spatial match in the decline of salmonellosis incidents and the trichomonosis epidemic mortality, it is possible that the epidemiology of the endemic bacterial pathogen was influenced by the emergence of the protozoal parasite in Great British finch populations. Greenfinches appear to have an important role, and it has been proposed that they might act as amplifier hosts, leading to pathogen spill-over to sympatric species during salmonellosis outbreaks (Pennycott et al., 1998a; Chapter 2). The reduced greenfinch breeding population might have led to reduced densities or group size in garden habitats, impacting the likelihood of faeco-oral transmission of salmonella bacteria. Whilst the greenfinch population decline was significant (Chapter 6), the species remained a common bird across the period 2005-2008 present in gardens across Great Britain (BTO 2010; Risely et al., 2009): consequently it seems unlikely that the population change alone was sufficient to cause the decline in salmonellosis.

The succession of the predominant *S.* Typhimurium phage type from DT 40 to DT56(v), and its establishment, offers the most plausible explanation for the decline in disease incidents. The epidemiology of salmonellosis in human and animal populations can be characterized by cycling of dominant or epidemic phage types (Anderson 1968; Threlfall et al., 1978). Continued surveillance of passerine salmonellosis might identify emergent *S.* Typhimurium phage types likely to cause increased mortality in the future.

Characterisation of *S.* Typhimurium isolates collected from wild birds in northern England using pulsed-field gel electrophoresis (PFGE) was performed and included a subset of the passerine *S.* Typhimurium isolates from this study (Hughes et al., 2008). The study found that the 23 *S.* Typhimurium DT56(v) isolates examined were clonal on the basis of identical PFGE patterns. Only 3 *S.* Typhimurium DT40 isolates were available for study and these, although closely related, grouped in 2 PFGE patterns. Further characterization of passerine *S.* Typhimurium isolates on a national scale over a long-time scale is required to investigate the epidemiology of this disease and the degree

to which these bacterial strains are host-adapted. In particular, due to their geographical segregation it will be interesting to determine whether the DT40 isolates from south-western England are identical to those from Scotland (Figure 4.9a). PFGE studies have been undertaken on over 200 *S.* Typhimurium isolates collected from passerines from across Great Britain in this study and on those available from the preceding period (Chapter 2) and analyses are underway.

It was not possible to differentiate asymptomatic carriage and early clinical disease in the suspected carrier birds, however, greenfinches accounted for a third of these cases. In order to fully investigate the hypothesis that greenfinches play a central role in the epidemiology of salmonellosis, further studies screening live birds for evidence of enteric carriage in liaison with licensed ringers are required. Refsum et al. (2003) performed a similar study of Norwegian passerines and found enteric carriage to be most common in bullfinches, the species in which clinical disease was observed: c. 2000 birds were screened in order to identify the low prevalence (2%) of infection, therefore planned studies in Great Britain need to be large in scale. Prospective studies should be performed at sites with both a known history of salmonellosis and no history of disease. Site sampling would include PME of any birds found dead to confirm the cause of mortality incidents, microbiological sampling of bird tables as with Pennycott et al. (2002, 2005b) and live bird sampling using mist nets in garden habitats throughout the calendar year, both during and following the salmonellosis season (Sept – March inclusive).

Colibacillosis

E. coli O86 infection was confirmed in the same finch species as reported by Pennycott et al. (1998a). Whilst a large number of non-finch species were examined, none of the non-lactose fermenting or late lactose fermenting *E. coli* isolates from this group were confirmed as serogroup O86. Siskins were the species in which the infection was most commonly confirmed and the geographical distribution of cases in Scotland, where the siskin population has greatest density, indicates that the siskin may play an important

role in the disease ecology. To further investigate the epidemiology of *E. coli* O86 infection, screening of live birds for asymptomatic carriage using the model created by Grant et al. (2007) with an appropriate selective media and microbiology protocol, as advised by Pennycott et al. (2006), is required. Where *E. coli* O86 infection was considered the sole COD, a similar seasonal trend was seen in the cases from across Great Britain, which peaked in the late spring months, and cases from Scotland (described by Pennycott et al., 2002), which occurred between March and May.

E. coli O86 infection has not been reported in passerine species outside Great Britain. *Escherichia albertii* was described as a cause of epidemic mortality in redpoll finches (*Carduelis flammea*) in Alaska, in the winter of 2004, and Oaks et al. (2005) noted the similarity of this outbreak to *E. coli* O86 infection in Scottish birds. Affected redpolls were in adequate body condition, indicating an acute disease progression, and had signs of mild enteritis on PME, sometimes in combination with discoloured intestinal contents. Non-lactose fermenting *E. albertii* was isolated in pure culture from multiple birds; the isolate identity was confirmed by 16S rRNA gene sequencing and PCR detected presence of the genes coding for intimin and cytolethal-distending toxin.

Within this study, the number of cases and incidents where *E. coli* O86 infection was confirmed was much smaller than that for salmonellosis and trichomonosis indicating that the disease may be less likely than these other diseases to be a significant cause of population level decline. Whilst a small number of sites reported high mortality of susceptible species thought to be due to *E. coli* O86 infection, the majority reported <5 dead birds.

In this study, 60% of affected birds were in normal body condition, a similar observation to Pennycott et al. (1998a). A significant sex bias was observed, with males more frequently affected than females, for all species and the siskin alone. This gender skew was reported in the study by Pennycott et al. (1998a), where the infection was found in 33 male and 10 female finches, and was observed in greenfinches affected by salmonellosis also (Chapter 2 and see above). Potential explanations for this gender bias

include differential susceptibility or exposure, due to physiological or behavioural differences (Chapter 2). The majority of affected birds were adults, although cases were recorded in a nestling and juvenile birds. Since nestling and juvenile birds accounted for a relatively small proportion of the submissions, more research is required to accurately assess the importance of *E. coli* O86 infection as a cause of nestling mortality as reported by Pawiak et al. (1991). Cases where *E. coli* O86 infection was considered the sole COD had minimal gross abnormalities; abnormal discoloured intestinal contents were described in some cases and distension of the upper alimentary tract was present in a small number of cases, however, the majority of cases simply had minimal gizzard contents and autolysed intestinal tracts, frequently with scant contents. Diagnosis of *E. coli* O86 infection relies on microbiological examination with no pathognomonic features at PME.

Pasteurellosis

Pasteurella multocida infection was considered the COD in 14 birds of multiple species submitted from sites across Great Britain throughout the calendar year. The majority of birds had evidence of predation injury, such as puncture wounds, consistent with cat attack. *P. multocida* is the most commonly cultured bacterium from infected cat bite wounds in humans (Freshwater 2008). A recent survey of domestic cats in the U.S.A. cultured gingival swabs and found hyperendemic *P. multocida* carriage in c. 90% of the 409 pets tested (Freshwater 2008). Domestic cats are frequent predators of wild birds within garden habitats in Great Britain (Woods et al., 2003). The findings of this study are consistent with pasteurellosis in garden birds as a secondary consequence of cat predation, rather than a primary pathogen of passerine species circulating in wild bird populations as with avian cholera epizootics in waterfowl and water birds caused by different strains of the bacterium (Samuel et al., 2007). Mortality estimates of passerines due to cat predation should take morbidity and mortality caused by secondary infection of bite wounds into account.

Yersiniosis

Yersiniosis was confirmed in a variety of finch and other garden bird species. Mortality incidents occurred sporadically at a low incidence predominantly during the colder months of the year. Whilst *Y. pseudotuberculosis* appeared to act as a primary pathogen in several of the affected birds, significant concurrent disease was present in some individuals. Single birds were typically reported to be affected; where multiple birds were affected concurrent significant disease with pathogens frequently associated with mass mortality was often present. Yersiniosis incidents were widespread affecting birds in Scotland, Wales and England, although the latter cases were restricted to the southern central and western counties; whether this represents a meaningful geographical cluster is unclear given the small number of cases.

***Suttonella ornithocola* infection**

Members of the Paridae, and to a lesser extent the Aegithalidae, are common birds in garden habitats in Great Britain, particularly those with feeding stations (Toms 2003), and their breeding populations are widely distributed across the country (Risely et al., 2008).

In this study, *S. ornithocola* infection was diagnosed from 4 incidents of mortality involving members of the Paridae between 1st April 2005 and 31st March 2008. One of these incidents also involved morbidity of long-tailed tits (Aegithalidae). Prior to this study, incidents of tit mortality associated with *S. ornithocola* infection had been demonstrated in Britain only in 1996 (Kirkwood et al., 2006) and the bacterium has not yet been reported from other countries.

Infected birds usually were in thin body condition and, in all cases, the appearance of the alimentary tract suggested that they had been anorexic prior to death. Poor plumage condition was noted in each case, suggesting that the birds had not been preening adequately for a period prior to death, consistent with the observed clinical signs of non-specific malaise. Kirkwood et al. (2006) also reported clinical signs as being non-

specific, including fluffed-up plumage, lethargy and reduced awareness. In addition, in separate incidents, these authors reported gaping in birds, as if they were trying to swallow, and showing excessive thirst, but such signs were not observed in the current study.

On PME, gross congestion of the lungs was present in 2 of the 4 birds examined and this observation was made in 4 of the 21 birds examined by Kirkwood et al. (2006). In 1 of the 2 birds for which the lung was examined microscopically, histopathological examination demonstrated acute necrotising pneumonitis associated with Gram-negative rods, morphologically consistent with *S. ornithocola*. A mixture of Gram-negative and Gram-positive rods was found in the air spaces of the second case examined in combination with pulmonary congestion and/or oedema. No abnormalities were found in the other tissues examined histologically.

Suttonella ornithocola was cultured from the lung in each infected tit and from the liver in 1 of the 4 cases. No hepatic lesions were detected on gross or histopathological examination. Post-mortem bacterial overgrowth might explain culture of the bacterium from multiple sites in these tits, although disseminated infection and bacteraemia could have occurred before death. No evidence of *S. ornithocola* infection was found in tits that died of trauma or predation during the study period. The results of this study support the hypothesis that *S. ornithocola* is a causative agent of pulmonary disease in tits and that disease outbreaks caused by this bacterium occur at a low incidence.

Suttonella ornithocola-associated mortality incidents all occurred in April: this is consistent with the temporal cluster of cases reported by Kirkwood et al. (2006) which all occurred between March and May. Kirkwood et al. (2006) hypothesised that this apparent seasonality occurs because the pathogen is spread in late spring and early summer, possibly facilitated through the arrival of summer migrants. In the spring months, increased contact rates between birds might occur, for example associated with breeding activity.

In the current study, *S. ornithocola* infection was diagnosed only in birds beyond their post-juvenile moult, categorised as adult, although it was not possible to differentiate between first year and adult birds on the basis of available plumage records. Future research should clearly discriminate whether birds are typically affected by *S. ornithocola* infection in their first year of life or also in subsequent years. All birds with *S. ornithocola* infection in this study were male. A similar skewed sex ratio was seen in 1996, where 14 of the 16 birds examined were male, although the reason for this, or even if it represents a true trend, is not known. It is plausible that gender-related behavioural differences during the breeding season might explain the bias toward males observed so far (Bouwman et al., 2010). Kirkwood et al. (2006) noted that exposure to a common risk factor also might be involved and that all affected birds were in provisioned gardens, as was the case in the current study.

Mortality incidents associated with *S. ornithocola* infection were confirmed in England and Wales, indicating that this bacterium is geographically widespread in Great Britain. Similarly, there was no evidence of geographical clustering in the incidents reported by Kirkwood et al. (2006), which occurred across 10 counties in England and Wales.

As with 10 of the 11 incidents reported by Kirkwood et al. (2006), mortality of blue tits was reported in all *S. ornithocola* incidents in this study, although concurrent morbidity and/or mortality of other Paridae or Aegithalidae species were reported in all but 1 of the incidents (Table 4.4). This might suggest that blue tits play a role in the epidemiology of the infection, or are particularly susceptible to *S. ornithocola* infection.

The current study has shown multiple, sporadic incidents of tit mortality associated with *S. ornithocola* infection over a 3-year period. This pattern suggests that *S. ornithocola* infection might be endemic within native tit populations and contrasts with the findings in 1996 where a temporal cluster of 11 incidents was seen over a period of 3 months (Kirkwood et al., 2006). The mechanism of transmission of *S. ornithocola* is currently unknown, but as the infection is associated with acute necrotising pneumonitis, aerosol transmission is a likely route of spread.

Further investigation is required to enhance our understanding of the epidemiology and significance of *S. ornithocola* infection in birds. This study suggests that this bacterium is an endemic, primary pathogen of tit species in Great Britain, causing disease outbreaks at a low incidence across the country with a seasonal (spring) trend. Continued monitoring to increase the number of confirmed incidents will help to determine whether the gender skew towards males and the apparent predisposition of the blue tit to disease found in this study, and in a previous study by Kirkwood et al. (2006), are true.

Avian tuberculosis

Whilst avian tuberculosis has been confirmed in species from at least 15 avian orders, disease is most commonly seen in Gruiform, Galliform and Anseriform species and occasionally in Passeriform species (Friend 1999b). Only a single case of mycobacteriosis was confirmed in this study of >1,500 birds involving a single adult wood pigeon: culture for species identification was not performed. Keymer (1997) highlighted the potential role of wood pigeons in the spread of Johne's disease (*Mycobacterium avium paratuberculosis*) infection to cattle, based on experimental infection work by Collins et al. (1985), which indicated that wood pigeon *Mycobacterium* sp. isolates were distinct and pathogenic to both calves and chickens. Darkening or dulling of the plumage, as has been previously reported in wood pigeons with avian tuberculosis, was not noted in this case (Friend 199b). Whilst birds with avian tuberculosis typically have gross visible lesions exceptions can occur (Friend 199b). Since the protocol in this study did not include histopathological examination of all tissues as routine, or mycobacterial culture, it is possible that tuberculous infection not associated with gross lesions may have been missed in some birds.

Aspergillosis

Aspergillosis was a sporadic cause of mortality in individual birds in this study. Respiratory tract lesions were most common, typical of the disease in wild birds and

reflecting the route of infection through inhalation of the ubiquitous fungal spores (Converse 2007). Free-living waterfowl, gulls and corvid species are most frequently affected by aspergillosis: songbirds are occasionally affected (Friend 1999c) and this is reflected in the relatively small number of cases in this study. Birds with chronic aspergillosis are frequently immunocompromised (Converse 2007; Cacciuttolo et al., 2009): this was observed in this study where birds with aspergillosis suffered from concurrent infectious disease, were immunologically näive juveniles and a single bird which was submitted to a rehabilitation centre and briefly housed in captivity. No incidents of acute aspergillosis with multiple mortality events following consumption of heavily contaminated foodstuffs, such as those which have occurred in the U.S.A. caused by mouldy silage (Neff 1955) or corn (Bellrose 1945), have been reported in Great Britain or were found in this study.

Focal pulmonary or air sac lesions were most typical, as commonly described in wild birds (Friend 1999a). A single wood pigeon had a tracheal aspergilloma which occluded the primary airway leading to death by asphyxia. Inhaled grain within the upper respiratory tract has been proposed to act as a nidus for aspergilloma formation (Converse 2007): histopathology was not performed on the lesion to evaluate whether this might have been the case in this bird. Tracheal aspergillomas have been less frequently reported in wild birds than captive psittacines but cases have occurred, for example in waterfowl (Stroud et al., 1982) and crane species (Converse 2007).

Trichomonosis

The impact of trichomonosis on British finch populations (Chapter 6), comparison of the epidemic mortality caused by trichomonosis that occurred in 2006 and 2007 (Chapter 7), molecular epidemiology of trichomonosis in British garden birds (Chapter 8) and the likely mechanism of parasite spread to continental Europe (Chapter 9) are discussed in this thesis. Columbiform trichomonosis cases had characteristic pathology with caseous pharyngitis in all cases. Hepatic lesions, as observed in a single bird, have been previously reported with certain *T. gallinae* strains in feral pigeons (Perez-Mesa et al., 1961; Cole 1999a).

Coccidiosis

Gregarious species were principally affected by coccidial infection in this study: since protozoal parasites have an intestinal life-cycle with faeco-oral route of transmission, congregation at feeding stations with poor hygiene might predispose to increased oocyst exposure.

The isosporan parasites (*I. lacazei, I. fringillae and I. chloridis*) confirmed in the subset of house sparrow, chaffinch and greenfinch samples from this study that were examined have been widely reported in passerines from Great Britain (Yakimoff et al., 1938; Anwar 1966). These coccidian parasites have an intestinal life cycle and their pathological significance in wild birds is unclear. Pennycott et al. (1998a) considered the infection to be an incidental finding in Scottish finches. However, *I. lacazei* infection has been experimentally shown to compromise carotenoid levels in the plumage of male greenfinches and to cause transient weight loss and reduced serum parameters (e.g. triglyceride, albumin and vitamin E); protozoal enteritis and subsequent malabsorption was proposed as the likely mechanism for these changes indicating that serious disease can result from this parasite infection.

Atoxoplasma sp. have also been confirmed in British greenfinches (Ball et al., 1998). These apicomplexan parasites have extra-intestinal life cycle stages and have been associated with clinical disease known as 'going light' in captive greenfinches (Cooper et al., 1989). Differentiation of *Isospora* and *Atoxoplasma* spp. is problematic since their oocysts are very similar in appearance (Pennycott et al., 1998a). Histopathological examination of the liver and spleen, examination of blood films and tissue impression preparations is necessary to confirm atoxoplasmosis but this was not performed on the coccidiosis cases in this study. A recent North American study of passerine isosporoid coccidia from the Americas, Asia and Africa concluded that they were monophyletic and most closely related to *Eimeria* spp. (Schrenzel et al., 2005): a similar study incorporating European samples would be of interest.

Quantification of the severity of the infection through subjective (as in this study) or objective oocyst counts is problematic. Oocyst shedding has been shown to vary with a number of factors, for instance, diurnal variation in output occurs, with peak shedding in the late afternoon (Brown et al., 2001). Real-time PCR has been used for quantification of *Eimeria* spp. infection in chickens (Morgan et al., 2009). A nested PCR based on the 18S rRNA gene of *Isospora* spp. was used to confirm *Atoxoplasma* spp. infection in zoo birds: whilst the PCR was not specific, the diagnosis was made for birds with positive amplification from liver or splenic tissue since *Isospora* spp. are restricted to the intestinal tract (Adkesson et al., 2005). There is a need to develop a sensitive and specific PCR for *Atoxoplasma* spp. in the future that can be used on faecal samples.

It is interesting to note that the birds with coccidial infection in this study were frequently immunocompromised with concurrent disease or were immunologically näive juveniles. Concurrent disease might enable significant disease to develop from an asymptomatic or subclinical infection with these parasites.

Helminthosis

Helminth parasites were frequently observed in blackbirds and starlings in this study: published studies have reviewed the parasite species in these bird species in different countries (Boyd 1951; Misof 2005). Species identification was not possible as part of this study for the gastrointestinal parasites: future collaboration with Dr E. Harris at the Natural History Museum is planned as part of the British Parasite Biodiversity Project.

Differentiation of clinically significant and incidental parasite burdens is problematic and can only be achieved subjectively through appraisal of a number of factors, principally body condition and evidence of concurrent disease. Subclinical health impairment might result from low parasite burdens considered incidental in these cases.

The blackbird and starling diet includes a large proportion of invertebrate food items: many helminth parasites have indirect life cycles (e.g. *Syngamus trachea,* acanthocephalan species) with invertebrate intermediate or paratenic hosts (e.g.

earthworm) which explains why these species are those commonly infected (Cole 1999b, 1999c) amongst garden birds.

Avian pox infection

Avian pox infection was confirmed as a sporadic COD in non-Paridae species in which the disease has been previously reported in Great Britain, namely the house sparrow, wood pigeon and dunnock. Typically single birds were affected at each site. Cases were confirmed in each year of study and from a wide distribution across Great Britain. These findings are consistent with endemic avian pox infection in non-Paridae garden bird species in Great Britain. Affected birds were in poor body condition and avian pox was considered a significant contributory factor to the COD. However, reports of observed skin lesions consistent with avian pox in these species were received from members of the public (GBH*i, unpublished data*) where the birds did not appear adversely affected by the lesions. Collaboration with ornithologists and licensed ringers, in addition to the general public, offers the best approach to accurately determine the prevalence of avian pox infection in non-Paridae species where the case mortality rate appears to be low.

In the late summer of 2007, severe cases of avian pox infection were observed for the first time in Great Britain in Paridae species, principally great tits, with florid lesions on the head and body. Lesions were frequently large (<20 mm diameter) with a caseous cream or yellow-coloured caseous core on cut tissue: the appearance was very similar to that described with avian poxvirus infection in the same species in Germany (Holt et al., 1973).

In recent years, Gruber et al. (2007) reported a single avian poxvirus incident involving great tits at a garden site in Vienna, Austria, 2005. These authors observed a similar distribution of extensive lesions restricted to the head in affected birds and considered that the case mortality rate was high. Similarly, nodular lesions restricted to the head and eyelids were observed in great tits with avian poxvirus infection in Hungary: the disease was found at low prevalence (0.8% - 15/1819 birds) in this study of birds caught by licensed ringers (Palade et al., 2008).

The disease significantly impacted affected tits in this study, impairing their ability to avoid predators and feed normally. Multiple birds were typically affected at each site. The incidents were clustered in time and space with the majority occurring in south-east England between July and October 2007. These findings are consistent with a regional outbreak of avian pox infection representing an emerging infectious disease of English Paridae species. Host, vector or pathogen factors may have influenced emergence of this virus as a pathogen affecting Paridae species in Great Britain.

Host factors that might predispose to spill-over of infection include congregation and mixing of bird species at feeding stations. Avian pox infection outbreaks have been observed affecting house finches (*Carpodacus mexicanus*) at feeding stations in the U.S.A. (McClure 1989). The Breeding Bird Survey 2008 (Risely et al., 2009) indicates that the great tit population in England has increased by 43% between 1995 and 2007 which, coupled with the frequent use of feeding stations (Chamberlain et al., 2005) by this species, might have influenced pathogen transmission dynamics.

Mosquitoes are considered an important biting insect vector for avian pox infection (van Riper et al., 2007). Over thirty mosquito species have been reported in Great Britain, including several ornithophillic species such as *Culex pipiens* (Higgs et al., 2004). The summer of 2007 in England had average temperature (mean temperature anomaly 0.0 °C as compared with 1971-2000) but high rainfall (mean rainfall anomaly 177% as compared with 1971-2000), which could have favoured mosquito populations (Met Office 2010). No national monitoring system for mosquito populations in Great Britain was in place in 2007. However, a marked rise in the number of mosquito bites in humans was received by NHS direct in 2007 suggesting an increased mosquito population: similar changes in ornithophillic mosquito species such as *Culex pipiens*, which utilise container habitats (e.g. water butts in gardens), are likely to have occurred (J. Medlock, *pers. comm.*). The relative importance of mosquitoes as viral vectors when compared with mites, midges and flies in Great Britain is unknown. However, great tit disease incidents typically occurred between July and September, i.e. during the peak

mosquito season in temperate climates. Similarly, the relative importance of indirect and direct transmission routes is unknown.

Introduction of a novel avian poxvirus, or genetic mutation of an existing strain, might have occurred. Whilst great tits were most frequently affected, infection was also suspected in the blue tit indicating that the virus is not species-specific: the species range susceptible to infection is unknown. Virus isolation using chorio-allantoic membranes and experimental challenge studies would be required to assess the range of species susceptible to the virus and the severity of resultant lesions (Tripathy et al., 2000).

The restricted geographical range of Paridae incidents was not matched by incidents in non-Paridae species: this finding is most consistent with an independent strain of virus affecting Paridae and non-Paridae species, however, further research and surveillance is needed to evaluate this hypothesis. No recent increase in avian pox infection has been reported in captive, zoo or wild bird species.

Molecular epidemiological studies are required to investigate whether the Paridae cases are caused by a novel clonal virus strain and to assess the diversity of avian poxviruses circulating within wild bird species in Great Britain. Phylogenetic analyses based on avian poxvirus core 4b gene sequence have been performed on birds from across Europe (Lüschow et al., 2004; Weli et al., 2004ab) and the U.S.A. (Adams et al., 2005). Gyuranecz et al. (2007) recently described differentiation between avian poxvirus strains using sequence analysis of the polymerase gene. A combination of these published techniques could be used on avian pox isolates from Great Britain.

Comparison of a partial nucleotide sequence from the avian poxvirus core 4b gene was 100% identical between the Norwegian (Weli et al., 2004ab) and Austrian avian poxvirus strains collected from great tits (Gruber et al., 2007). It would be particularly interesting to compare the British avian poxvirus strain with those from continental Europe to assess the degree of conservation for this host species across regions.

Drowning or hypothermia

Drowning is an infrequently reported COD in wild birds, presumably since they frequently practice water bathing to maintain plumage condition and are able to fly. Where drowning incidents occur they typically involve individuals rather than multiple mortalities, e.g. juvenile barn owls (*Tyto alba*) drowning in livestock water troughs (Ramsden 2003).

A retrospective review of mortality incident reports received by the IoZ since 1993 indicates that the starling mortality event in this study was not an isolated event. One incident was reported in May 1994 and 2 in May 1995 in garden ponds (n=2) and a swimming pool (n=1) where multiple starlings (18-33 birds) were found dead in the water body. The VLA investigated 2 events involving mortality of 23-41 juvenile starlings in 2006 in swimming pools in East Anglia and a third involving 18 starlings in a small ornamental pond in Sussex (VLA 2006). Following heavy rain, 706 dead and soaked starlings were found in a city park in Hull; it was thought that they had fallen from their communal roost during the storm and then succumbed to hypothermia (VLA 2007). There was no contemporaneous history of morbidity or mortality affecting other domestic or wildlife species at these sites. With the exception of the Hull park, no sites were reported to have large roosts of starlings over the water body which excluded the possibility that birds died in the trees and then fell into the water body after death. PMEs, where possible, found no evidence for infectious, toxic, or traumatic aetiologies and drowning, or hypothermia secondary to waterlogging, was considered the COD.

Where details were available, the man-made water bodies had sharp sides extending above the water level and no floating objects or materials breaking the surface of the water. Consequently, waterlogged birds may have found it impossible to exit these water bodies as compared with natural ponds or streams with gradually sloping sides.

Juvenile starlings were typically involved in these drowning incidents. Juvenile starlings leave the nest from around 21 days and become fully independent from their parents around 12 days later (Feare 1984). During this period, juvenile starlings are particularly

garrulous and gregarious, frequently observed feeding and bathing in flocks. This period has been described as high risk, whilst they learn to become independent from their parents, when they are more likely to fall prey or become victims of accidents such as "*falling into ponds, hitting windows, or road traffic accidents*" (Feare 1984).

A behavioural explanation relating to the strong flocking behaviours of starlings seems most likely. The cryptic dull grey plumage of starlings, present until the post-juvenile moult which typically commences in early July in first broods, may be particularly vulnerable to waterlogging and this should be investigated. Considerable financial and logistical investment is given to investigation of multiple mortality events in wild birds, principally due to concerns of zoonotic notifiable disease events: drowning or hypothermia secondary to waterlogging of plumage should be considered as a differential diagnoses for mass mortality events in starlings.

Oesophageal obstruction

Oesophageal obstruction was a novel COD in 2 adult birds in this study. Both the robin and dunnock have a catholic diet, but are chiefly insectivorous species (Toms 2003) not typically thought to feed on peanuts provided at garden feeding stations. It is possible that these individual birds, for some unidentified reason, had difficulty in obtaining sufficient natural diet items and were attracted to the readily available food items. Alternatively, the novel food items may have been taken inadvertently. Large food boluses may be taken by these species, for example invertebrate larva or fruit; however these have a high water content and are easily deformed making them possible to swallow. In contrast, the hard peanut fragments in these 2 birds became obstructed at the level of the furcula.

The risk of choking on peanut fragments has been raised in a study which found dead great tit nestlings with peanut fragments within their gullet on 3 occasions. Whether these birds had indeed 'choked' on the peanut, or whether it was obstructed in the oesophagus is unclear (Cowie et al., 1988). This led the RSPB to advise that whole peanuts only be provided in wire mesh feeders for birds (RSPB 2009) and the bird food

industry to make peanut granules available. This report of oesophageal obstruction with peanuts in adult birds indicates that this current advice is worthwhile: however only 2 cases were found in this study and this event appears unusual.

Tick-related syndrome

The cases of TRS in this study were typical of the previous reports of the condition in the UK (Monks et al., 2006; Gould 2007) and France (Chastel et al., 1981). The collared dove is the species most frequently affected in England and Wales (Gould 2007) and a single TRS case was seen in this species in this study. A recent 4-year study found 3 TRS cases in free-ranging passeriform birds (Monks et al., 2006): whether these included the house sparrow and greenfinch, as in this study, is unknown as the species were not listed. Garden bird species which are predominantly ground-feeding might be predicted to be most commonly affected by TRS: this behaviour would increase proximity to tick habitats, for example hedgerows and bushes.

The TRS cases each conformed to the typical syndrome presentation of good body condition and peracute death with severe localized oedema and haemorrhage on the head, an area inaccessible to preening, extending from the site of tick attachment. Adult female *I. frontalis* ticks were found in each case, as with 98.4% of adult ticks in the study by Monks et al. (2006). *I. frontalis* is an ornithophillic 3-host tick with 1 generation per year (Doby et al., 1997). The cases occurred during August and September, the peak months for the condition in Britain in other studies (Monks et al., 2006; Gould 2007). This matches the period of peak feeding activity by the *I. frontalis* adult female tick (Doby et al., 1997).

TRS cases have been chiefly reported in East Anglia (Gould 2007), south-west England and south Wales (Monks et al., 2006). Both studies noted that their reliance on opportunistic submissions might have influenced this apparent geographical distribution in southern Britain. The current study, which for the first time incorporates a systematic surveillance element, found a similar distribution of TRS cases. The distribution of *I. frontalis* is thought to be restricted to southern England (Martyn 1988) therefore the

range of TRS cases appears to be dictated by parasite rather than host or environmental factors.

Underreporting of TRS cases is likely to occur, where birds are not submitted for pathological examination, since externally the clinical signs may be confused with acute trauma. Gould (2007) found the reason for presentation of collared dove casualties at a rehabilitation centre confirmed with TRS was head trauma or eye injury in the majority of cases: only 6% of birds were submitted with parasite infection as the primary presenting problem. On PME, the lesions of TRS contrast with those sustained by collision injuries or blunt trauma. Nevertheless few cases were confirmed in this study, which would support sporadic events occurring at low incidence.

The aetiology of TRS is unknown: no evidence for *Borrelia burgdorferi* sensu lato, *Bartonella* species, *Babesia* species and *Ehrlichia* species was found on PCR of tick and tissue samples in Britain (Monks et al., 2006). Idiosyncratic host response to tick exposure has been proposed as a potential mechanism (Monks et al., 2006). The most plausible hypothesis is that toxins are produced in the tick saliva with anti-coagulant and tissue-toxic properties (Monks et al., 2006). The discrete margin between frank haemorrhage and normal tissue observed in the collared dove TRS case in this study (Figure 4.27) seems most consistent with this hypothesis: local toxin infiltration and distribution from the site of the tick bite could account for this unusual and striking appearance. Histopathological evaluation of cases might help elucidate the mechanism of action further.

COD not established

Overall, the COD category was not established for 11% of the birds examined in this study which is slightly lower than the percentage of unresolved cases reported by Jennings (1961) of 17% (171/1005 birds) and Keymer (1958) of 18% (24/132 birds). In many cases, the advanced degree of decomposition would have limited interpretation and led to the inconclusive PME findings. However, it is probable that infectious disease, and other diagnoses, were missed in some cases. A major limitation of this

study was the small proportion of birds in which comprehensive histopathological examination was performed due to time and financial constraints. Consequently diseases for which histopathology, or other diagnostic tests not included within this standardised protocol, are required for diagnosis may be under-appreciated. For example, PCR screening of the tissue archive for evidence of *Chlamydophila psittaci* infection, in combination with histopathological examination, would be useful to appraise the importance of this potentially zoonotic pathogen, known to occur in British passerines, in the future (Simpson et al., 1989; Pennycott et al., 2009). Similarly, nutritional and toxic diseases may be under-diagnosed since additional expensive laboratory investigations (e.g. HPLC), not included in our routine examination protocol, would be required for their diagnosis. The blue tit was the species for which COD category was most frequently undetermined which might simply relate to its small body size and rapid autolysis: however it is tempting to speculate that cases of *S. ornithocola* infection, a fastidious bacterium to culture, may have been missed in some cases particularly since lung was not cultured routinely from Paridae submissions at all diagnostic centres.

CHAPTER 5: PATHOLOGICAL LESIONS OF SALMONELLOSIS IN THE HOUSE SPARROW AND GREENFINCH

5.1 INTRODUCTION

In Great Britain, the greenfinch (*Carduelis chloris*) and the house sparrow (*Passer domesticus*) are the species in which salmonellosis has been most frequently reported (Kirkwood et al., 1997; Pennycott et al., 1998a) and the results of this study support this apparent species predisposition (Chapter 2 & 4). Characteristic gross lesions have been described in passerine salmonellosis, principally involving the upper alimentary tract, liver and spleen (Daoust et al., 2000; Pennycott et al., 1998a). Few comprehensive histopathological studies have been performed on passerine salmonellosis. Refsum et al. (2003) described severe hepatic and splenic necrosis, sometimes in association with Gram-negative bacterial colonies with inflammatory cell infiltrate, and also acute foci of necrosis with no inflammatory response, in 9 Norwegian passerine species with salmonellosis. Daoust et al. (2000) described microscopic lesions in multiple organs, including the liver and spleen, with necrotic masses of inflammatory and parenchymal cells surrounded by large aggregates of inflammatory cells (mononuclear leucocytes and heterophils), fibrin and bacterial colonies, in 5 Canadian passerine species with salmonellosis. Neither study appraised the histopathological abnormalities for each species and their combined findings indicate some variation occurs in the chronicity of salmonellosis lesions with both acute and chronic granulomatous presentations.

Variable rates of disease progression have been shown to occur in passerine salmonellosis ranging from peracute mortality, for example in New Zealand where epidemic disease due to the novel *S.* Typhimurium DT160 occurred (Alley et al., 2002), to subacute/ chronic infection in Scotland and Norway where endemic infection with relatively predictable winter disease incidents occur (Pennycott et al., 1998a; Refsum et al., 2003). However, evaluation of interspecific variation in the lesions observed among passerine species commonly affected by salmonellosis in a single country and time

period has not been undertaken. Variation between species, if found, might inform our understanding of the reservoir of the infection.

In this chapter the post mortem findings of salmonellosis cases in the greenfinch and house sparrow are reviewed. Implications of these findings for the hypothesis that greenfinches may play an integral role in the epidemiology of the disease in Great Britain are discussed.

5.2 MATERIALS AND METHODS

Carcass submission

Between 1993 and 2004, opportunistic reports of garden bird mortality incidents were solicited from members of the public through a passive surveillance network including the British Trust for Ornithology (BTO), the Royal Society for the Protection of Birds (RSPB), the Universities Federation for Animal Welfare and the Zoological Society of London. Between 2005 and 2008, garden bird mortality incidents were recorded through the Garden Bird Health *initiative* (GBH*i*), combining opportunistic submissions through a passive surveillance network and systematic submissions through an active participant network of the BTO Garden BirdWatch (Chapter 3).

Post mortem examination (PME)

A network of regional diagnostic laboratories examined submissions from across Great Britain following a standardised PME protocol (Chapter 4). The species, age, sex, body mass and maximum tarsal length were recorded for each bird examined. Qualitative body condition scores ('emaciated', 'thin', 'normal', 'fat') were assigned based on visual inspection of pectoral muscle mass and fat deposits. The superficial pectoral muscles were removed and weighed and the maximum tarsus length was recorded in birds examined during the GBHi, 2005-2008. Quantitative body condition measurements were recorded (body weight/maximum tarsus length (BW: MTL) ratio and superficial pectoral muscle mass/maximum tarsus length (PM: MTL) ratio). Where the state of carcass preservation permitted, the liver organ weight and spleen dimensions were

recorded in birds examined during the GBH*i*, 2005-2008, and were used to subjectively identify hepatomegaly and splenomegaly. Where indicated, and where the degree of carcass decomposition permitted, samples were taken for microbiological, parasitological and histopathological investigations (Chapter 4). Cause of death categories were assigned in each case based on a review of all findings; these comprised 'infectious disease', 'predation', 'other trauma', 'other' and 'not established'.

Bacteriological examinations

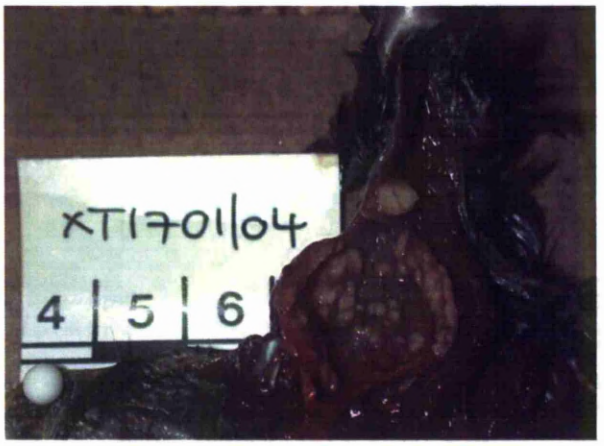
The liver, small intestine and/or crop/oesophagus, in addition to any lesions found, were routinely sampled aseptically, and examined for the presence of pathogenic bacteria using a standard protocol (Chapter 2 & 4). *Salmonella* spp. isolates were later submitted to the UK Veterinary Laboratories Agency (mid-nineties), to the Salmonella Reference Unit, Health Protection Agency (late-nineties to 2008), and the Scottish Salmonella Reference Laboratory, Stobhill (2005 to 2008), for biotyping (serotype and phage type) according to standardised international protocols (Anderson et al., 1977). Salmonellosis cases were defined on the basis of characteristic gross lesions and isolation of *Salmonella* spp. on direct culture in pure/ heavy growth from site(s).

Review of gross lesions in salmonellosis cases

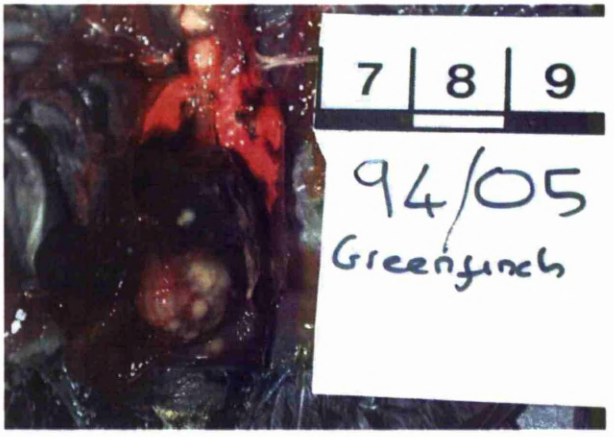
A retrospective review was performed on all 234 greenfinch and 74 house sparrow PME records from salmonellosis cases, 1993-2008. The upper alimentary tract, liver and spleen were the organs where abnormalities were most frequently observed on gross examination (Figure 5.1).

Figure 5.1: Gross lesions typically observed in passerine salmonellosis.

(a) Upper alimentary tract lesions in a greenfinch



(b) Hepatomegaly with focal lesions in a greenfinch



(c) Splenomegaly with focal lesions in a greenfinch

First, the gross lesions recorded in salmonellosis cases in the 2 species were compared between the study periods (1993-2003 and 2005-2008) to identify evidence for variation which might relate to recorder bias and to conclude whether consideration of the entire dataset for remaining analyses was sound. In addition, the proportion of salmonellosis cases with evidence of upper alimentary tract, liver and splenic abnormalities for both species combined was compared between cases with *S.* Typhimurium DT40 and DT56(v) infection to evaluate evidence for variation in lesions caused by these most frequently isolated phage types.

Upper alimentary tract lesions were classified as 'discrete', 'diffuse' or 'mixed' in appearance, each of the categories being mutually exclusive, and the frequency and proportion of birds within each category were compared between the greenfinch and house sparrow. Necrotic and fibrinopurulent foci, typically white, cream or yellow in colour (1-3mm diameter) with a variable, often firm, consistency were classified as 'discrete' lesions. The number of focal lesions ranged from single foci to miliary appearance. Classical 'target-like' lesions with a dark central core were described in a small number of cases. Thickening and necrosis of the crop or oesophageal mucosal surface, sometimes extending to involve the serosa, were classified as 'diffuse' lesions. Cases with focal lesions coalescing to plaques, or in combination with diffuse lesions were classified as 'mixed'. Details of the upper digestive tract contents were summarised.

Hepatic and splenic abnormalities were classified as organ enlargement (hepatomegaly and splenomegaly), focal lesions and perihepatitis/ perisplenitis; these non-mutually exclusive categories were the most frequent abnormalities described in these organs for salmonellosis cases. The proportion of cases in each category was compared between the greenfinch and house sparrow. Organ enlargement frequently occurred in combination with organ discolouration (dark and/or khaki colour) and abnormal tissue friability. Focal lesions were typically multiple pinpoint, white or cream-coloured (1-3 mm diameter) on the organ surface or scattered through the parenchyma. Perihepatitis/

perisplenitis was described for some cases, which referred to fibrinous adhesions between the organ serosa and neighbouring viscera.

Miscellaneous salmonellosis lesions were also summarised for the greenfinch and house sparrow.

Review of histopathological lesions in salmonellosis cases

Histopathological examination of the liver was performed on 56 greenfinches and 22 house sparrows with salmonellosis that presented between 2005 and 2008, as part of the GBH*i*. Cases were selected on the basis of the state of carcass preservation and where formalin-fixed tissue archives were available. Tissues were fixed in neutral-buffered 10% formalin, trimmed at the Institute of Zoology and stained with Haematoxylin and Eosin (Abbey Veterinary Services, Devon, UK). A limited archive of samples was available from birds examined between 1993 and 2003 and these were not included within this study.

Pathological lesions thought to be associated with salmonellosis were recorded as being either acute necrotic or chronic granulomatous lesions. Where lesions were equivocal, deeper sections were made to establish lesion type. In addition, liver tissue sections were scored according to the severity of hepatocellular fatty degeneration (Grade 0 – none, grade 1 – mild swelling of hepatocytes with a low number of intracellular vacuoles, grade 2 – swelling of hepatocytes with moderate numbers of intracellular vacuoles visible throughout the parenchyma, grade 3 – obvious marked vacuolation throughout the parenchyma) and bile duct proliferation (Grade 0 – none, grade 1 – more than 1 bile duct present in a few portal triads, grade 2 – more then 1 bile duct present in many portal triads, grade 3 – an average of over 2 bile ducts per triad).

Non-parametric analyses for categorical data (Pearson Chi-square test, Fishers exact Test) were used to compare the gross pathological and histopathological abnormalities between the greenfinch and house sparrow (Crawley 2002).

Body condition

Qualitative body condition scores and quantitative body weight variables (BW:TL ratio and PM:TL ratio) were examined by cause of death for the greenfinch and house sparrow. Data from birds that died as a result of 'other trauma', with no evidence of infectious disease, were used as a control group for comparison with the salmonellosis cases. Quantitative variables were tested for equality of variance between the salmonellosis and control group using the Levene's test and then compared using the parametric two-sample t-test or the Mann-Whitney U test as appropriate for parametric and non-parametric data respectively. Evidence for season (winter: Dec-Feb, spring: Mar-May, summer: Jun-Aug, autumn: Sep-Nov) as a confounding variable for analysis of the quantitative variable data was explored using the one-way ANOVA (Crawley 2002). SPSS 17.0 for Windows (SPSS inc., Chicago, USA) and R–CRAN (http://www.R-project.org) were used for the analyses. Data deficient cases were excluded from each analysis.

5.3 RESULTS

Comparison of gross abnormalities between datasets

No significant difference was found in the number of salmonellosis cases with upper alimentary tract lesions, or spleen abnormalities, between study periods (1993-2003 and 2005-2008), for either the greenfinch (n=234) or house sparrow (n=74) salmonellosis cases. However, the number of cases with liver abnormalities between study periods differed significantly for the greenfinch (X^2=12.316, df=1, P<0.001) and house sparrow (X^2=4.76 df=1, P=0.029), although the level of significance was marginal for the latter species. The binomial test to compare proportions found that the difference in greenfinches was largely accounted for by an increase in the proportion of cases with hepatomegaly in the more recent study period (X^2=9.230, df=1, P=0.002); although the proportion of cases with focal lesions in the more recent years was also greater at a lower level of significance (greenfinch X^2=5.783, df=1, P=0.02). The number of house sparrow salmonellosis cases that had liver abnormalities in the first study period was small (n=2), hepatomegaly accounted for all cases with liver lesions in the latter study

period (n=12). During the GBH*i* liver weights were taken, therefore recorder variation following improved examination technique offers the most plausible explanation for the apparent increase in salmonellosis cases reported with hepatomegaly, rather than an actual change in the lesions observed over time. Consequently all subsequent data analyses were performed for the combined data sets.

No significant difference was found between the proportion of salmonellosis cases with upper alimentary tract lesions (X^2=0.239, df=1, P>0.05), liver (X^2=0.608, df=1, P>0.05) or spleen abnormalities (X^2 =1.257, df =1, P>0.05) between *S.* Typhimurium DT40 and DT56(v) for the greenfinch and house sparrow data combined. All subsequent analyses include the combined dataset for all *S.* Typhimurium phage types.

Alimentary tract lesions

Lesions in the upper alimentary tract were the most frequently detected gross pathological abnormality in cases of salmonellosis and occurred in approximately equal proportions of the greenfinches (84% - 197/234 cases) and house sparrows (82% - 61/74 cases) examined. Where upper alimentary tract lesions were described in detail, focal lesions were most frequently observed in affected greenfinches (68% - 129/190 cases) as compared with a mixed presentation (19% - 36/190 cases) or diffuse lesions (13% - 25/190 cases). In contrast, in house sparrows, diffuse thickening of the upper alimentary tract wall was more frequently observed (51% - 31/61 cases) than focal lesions (31% - 19/61 cases) or a mixed presentation (18% - 11/61 cases). Pearson's chi-square test determined that there was a significant difference in the upper alimentary tract lesion categories between the 2 species (X^2=39.951, df=2, P<0.001). More specifically, the binomial test found that there was a significantly greater proportion of focal upper alimentary tract lesions (X^2=24.273, df=1, P<0.001) and a significantly lower proportion of diffuse lesions (X^2=35.645, df=1, P<0.01) in greenfinches than house sparrows. The proportion of salmonellosis cases with mixed upper alimentary tract lesions was similar for both species.

Where crop/oesophagus was sampled for bacteriology, salmonella isolates were obtained in the majority of cases, both of greenfinches (96% - 128/134 cases) and of house sparrows (94% - 49/52 cases). Observations on the volume of crop contents were noted in the majority of cases. For greenfinches and house sparrows combined, 57% of cases had empty or scant crop contents (92/165 greenfinches, 31/49 house sparrows) with the remainder having moderate to large volumes of crop contents (73/165 greenfinches, 18/49 house sparrows). Thickening of the terminal large intestinal and/ or cloacal mucosa, or lesions within the caecal tonsils, were described in 6 greenfinches and a single house sparrow from separate incidents.

Liver

Where comment on liver examination was available, liver abnormalities were noted in a significantly greater proportion of affected greenfinches (66% - 146/221 cases) than house sparrows (24% - 16/68 cases) (X^2=38.190, df=1, P<0.001). The majority of greenfinches with liver abnormality had focal lesions (75% - 109/146 cases), 51% (75/146 cases) showed hepatomegaly and 11% (16/146 cases) showed perihepatitis. Hepatomegaly was the most frequent abnormality described in house sparrows (75% - 12/16 cases) with salmonellosis that had liver abnormalities. The binomial test of proportions determined that focal lesions were found in a significantly greater proportion (X^2=9.927, df=1, P=0.002) and that hepatomegaly was observed in a significantly smaller proportion (X^2=10.688, df =1, P=0.001) of salmonellosis cases in greenfinches than house sparrows. Where liver samples were taken for bacteriological examination, a *Salmonella* sp. was isolated in 98% (211/216) of salmonellosis cases.

Spleen

The spleen was not located in a number of cases due to the small organ size and the degree of carcass decomposition. When the spleen was examined, however, splenic abnormalities were noted in a significantly greater proportion of greenfinches with salmonellosis (74% - 139/188 cases) than of house sparrows with salmonellosis (33% - 18/54 cases) (X^2=30.350, df=1, P<0.001). For cases with splenic abnormalities, splenomegaly was the most frequently observed abnormality in both greenfinches (94%

- 130/139 cases) and house sparrows (72% - 13/18 cases). Focal necrotic lesions were reported in a large proportion of affected greenfinches (71% - 98/139 cases) as compared with 33% (6/18 cases) of house sparrows with salmonellosis. For the cases with splenic abnormalities, a significantly greater proportion of greenfinches than house sparrows had evidence of splenomegaly (X^2=6.475, df=1, P=0.01) and focal lesions (X^2=8.254, df=1, P=0.004). Where samples of spleen were taken for bacteriological examination, a *Salmonella* sp. was isolated in 62 of 63 salmonellosis cases (98%).

Miscellaneous

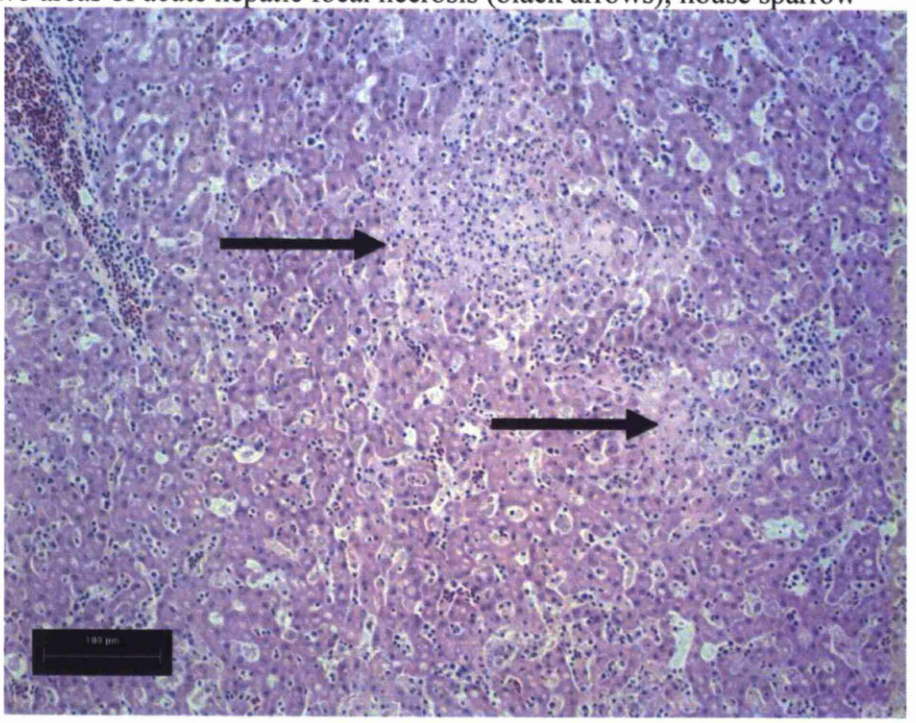
Pericarditis and/ or polyserositis, was observed in a small number of the greenfinches (n=6) and 1 house sparrow. Intraosseous lesions were described in the appendicular skeleton of 5 greenfinches and a single house sparrow.

Liver histopathology

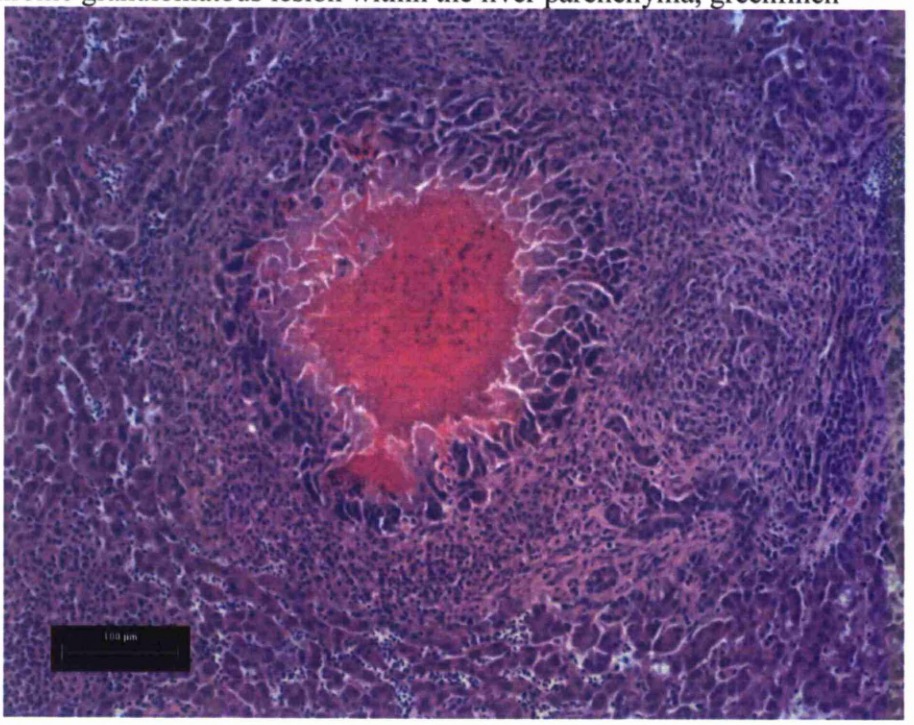
Chronic granulomatous lesions (Figure 5.2a) were found in 43 out of 56 greenfinch livers examined, whereas 12 greenfinches had acute foci of necrosis only (Figure 5.2b) and 1 greenfinch had no obvious lesions present. Of the 22 house sparrows examined, 5 had chronic granulomatous lesions and 17 were found to have acute foci of necrosis only. There was a significant difference in liver lesion type between affected species (X^2=20.583, df=1, P<0.001) with chronic granulomatous lesions more common in the greenfinch than house sparrow.

Figure 5.2: Histopathological abnormalities in the liver of birds with salmonellosis (H&E)

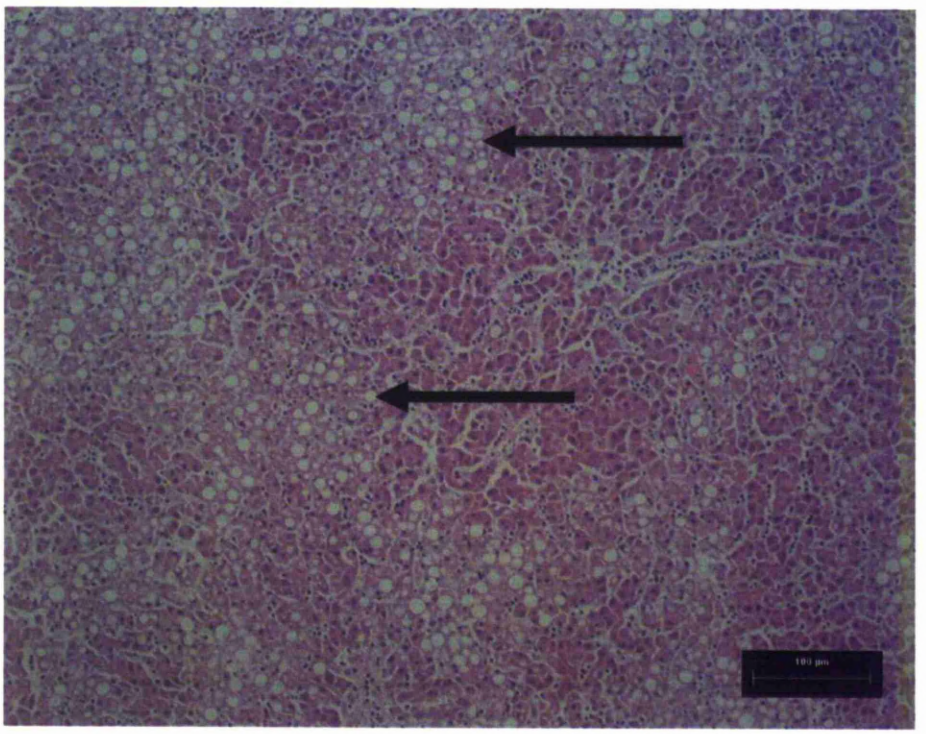
(a) Two areas of acute hepatic focal necrosis (black arrows), house sparrow



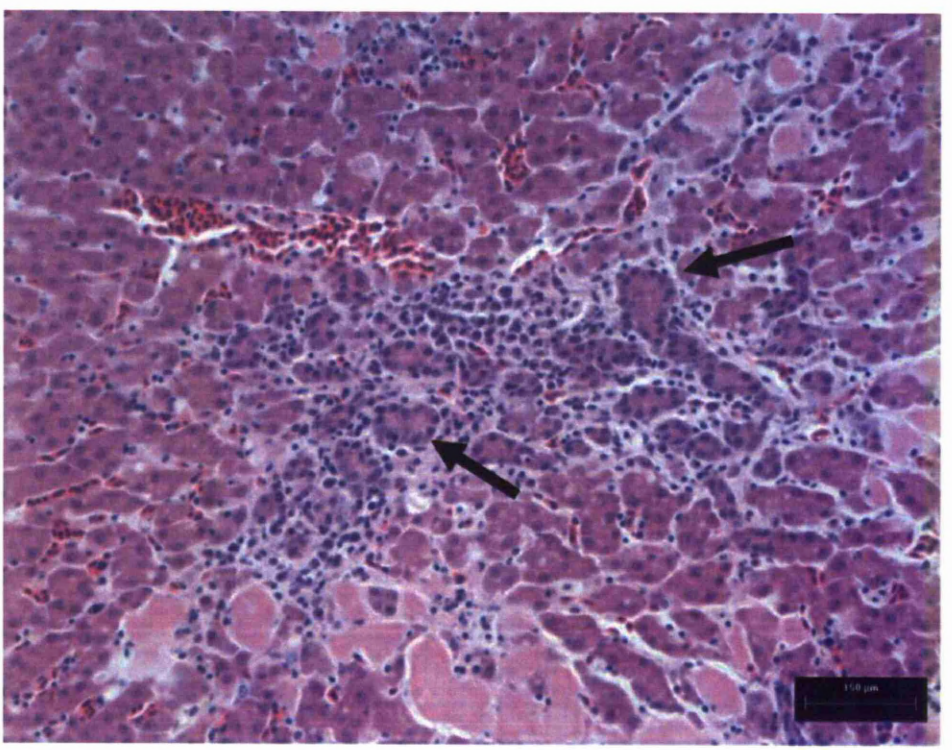
(b) Chronic granulomatous lesion within the liver parenchyma, greenfinch



(c) Marked hepatocellular fatty degeneration (black arrows) in a house sparrow



(d) Bile duct proliferation (black arrows) in a greenfinch



No significant difference was found in the hepatocellular degeneration (Figure 5.2c) scores between species (X^2=0.009, df=1, P>0.05) (Table 5.1). Bile duct proliferation (Figure 5.2d) scores were significantly different between greenfinches and house sparrows (X^2=17.149, df=1, P=0.000); a greater proportion of greenfinches with salmonellosis showed evidence of bile duct proliferation than house sparrows (X^2=14.820, df=1, P=0.0001).

Table 5.1: Hepatic histopathology scores for the greenfinches and house sparrows examined. For score criteria see Materials and Methods.

Score category	Hepatocellular degeneration		Bile duct proliferation	
	Number of greenfinches (%)	Number of house sparrows (%)	Number of greenfinches (%)	Number of house sparrows (%)
0	25 (44.6)	10 (45.4)	35 (62.5)	21 (95.4)
1	10 (17.9)	4 (18.2)	11 (19.7)	1 (4.6)
2	21 (37.5)	8 (36.4)	5 (8.9)	0
3	0	0	5 (8.9)	0

Body condition

Qualitative assessment of body condition categorised 41/225 (18%) greenfinches and 7/72 (10%) house sparrows as emaciated, 175/225 (78%) greenfinches and 55/72 (76%) house sparrows as thin and 9/225 (4%) greenfinches and 10/72 (14%) house sparrows as normal. The Pearson's chi-square test determined that there was a significant difference in the distribution of body condition scores between the species (X^2=10.790, df=2, P=0.005). Binomial test of proportions determined that although the proportion of greenfinches with salmonellosis which were emaciated was greater than that for house sparrows, the difference was not significant at the 5% level (X^2=2.315, df=1, P=0.13). The proportion of salmonellosis cases in thin condition were very similar for each species (X^2=0.007, df=1, P=0.94). However, the proportion of house sparrows with salmonellosis in moderate/ normal body condition was greater than that of greenfinches (X^2=7.333, df=1, P=0.007).

Quantitative measures of body condition were available for 29 adult greenfinches in the control group. One-way ANOVA found no statistically significant variation between

season for either the BW: MTL ratio (n=29 Levene's test, P>0.05, F=0.470, df=2, P>0.05) or the PM: MTL ratio (n=17 Levene's test P>0.05, F=0.380, df=2, P>0.05) within the control group, consequently season was not considered further as a confounding variable and the data were grouped for comparison with salmonellosis cases. Quantitative measures of body condition were available for only 6 adult house sparrows in the control group submitted across the seasons; the small sample size limited analytical comparison across the year and the dataset was grouped for comparison with the salmonellosis cases. Birds affected by salmonellosis had a significantly lower average value for quantitative measures of body condition than the control group for both the greenfinch (BW:MTL Levene's test P<0.001, Mann Whitney-U=846.5, P=0.000 and PM:MTL Levene's test P<0.05, Mann Whitney-U=100.0, P=0.000) and house sparrow (BW:MTL Levene's test P>0.05, t_s=-5.133, P<0.000 and PM:MTL Levene's test P>0.05, t_s=-5.081, P<0.000).

5.4 DISCUSSION

The upper alimentary tract, liver and spleen were the predilection sites for salmonellosis abnormalities in the greenfinches and house sparrows in this study. The appearance and organ distribution of the salmonellosis lesions were similar to other described cases of salmonellosis in passerine birds with multi-organ involvement and terminal septicaemia (Pennycott et al., 1998a; Daoust et al., 2000; Refsum et al., 2003). There was no evidence for an association between the distribution of lesions and the *S.* Typhimurium phage type (the most common phage types, DT40 and DT56(v), were assessed).

In both greenfinches and house sparrows with salmonellosis, upper alimentary tract lesions were the most frequently observed; an observation consistent with the majority of other studies (Daoust et al., 2000; Refsum et al., 2003). This suggests that upper alimentary tract lesions may develop early in the course of the disease in these species and that the infection may then spread to involve other organs. Explanations for the upper alimentary tract as a predilection site for lesions have been proposed. These include the crop as the initial portal of entry into the body, and as the site where

prolonged contact of contaminated food with the mucosa occurs (Daoust et al., 2000; Refsum et al., 2003).

Birds of both species with salmonellosis were frequently found to have a moderate to large volume of crop contents. Identifiable sunflower seed and peanut remains within the digestive tract of many cases indicated that these birds continued to visit and feed at garden feeding stations until shortly before death. At this time it is likely that birds were excreting large numbers of *S.* Typhimurium bacteria in their faeces and, hence, posed a threat of infection to sympatric susceptible species. A tendency for sick birds to stay in the vicinity of garden feeders has been reported previously (Kirkwood et al., 1995). Distension of the crop with large volumes of recently ingested food was reported in some cases and implies that organ stasis may occur in the later stages of the disease, either as a consequence of mechanical interference with peristaltic function, or due to lesion severity (and presumably, pain) inhibiting crop emptying (Kirkwood et al., 1998). Systemic ill-heath and generalised weakness might also be a factor influencing the rate of crop emptying.

Interspecific variation in the type or distribution of salmonellosis lesions in garden birds has neither been explored nor confirmed previously. Whilst no significant difference was found between species for the occurrence of upper alimentary tract lesions overall, focal lesions occurred in a greater proportion of greenfinches whilst diffuse lesions were observed in a greater proportion of affected house sparrows. Liver abnormalities were significantly more frequent in greenfinches than house sparrows and, where they occurred, lesions tended to be focal in greenfinches and diffuse in house sparrows. Splenic abnormalities were also significantly more common in greenfinches than house sparrows with salmonellosis and, in cases where they occurred, splenomegaly and focal lesions occurred in a greater proportion of greenfinches than house sparrows.

Histopathological examination of liver samples showed that salmonellosis results in the formation of areas of acute focal necrosis and chronic granulomatous lesions in both species. Focal hepatic necrosis has been recorded previously, in greenfinches, house

sparrows and chaffinches affected by salmonellosis (Routh et al., 1995; Alley et al., 2002). However, the majority of house sparrows examined in this study had areas of acute focal necrosis, with chronic granuloma formation being a rare finding. Conversely, granuloma formation was common among greenfinches. In all greenfinches acute necrotic lesions were also present, as would be expected with an ongoing bacteraemia. The fact that both types of lesion are seen in each species but in significantly different frequencies suggests that disease progression in house sparrows is limited whereas the disease continues to progress in greenfinches resulting in the development of chronic lesions associated with the bacterial infection.

Hepatocellular fatty degeneration was observed in just over half of the greenfinch and house sparrow salmonellosis cases with no significant variation between the species. Hepatocellular fatty degeneration is observed with subacute to chronic disease presentations; consequently it is not unexpected for both species to be affected. However, bile duct proliferation was seen relatively frequently in greenfinches whereas it was only recorded in a single house sparrow that also had chronic granulomatous lesions. As bile duct proliferation is a chronic change occurring in response to obstruction of bile outflow (Randall et al., 1996) this could also be a useful indicator of the relative rates of disease progression in each species, supporting the conclusion that greenfinches typically experience a more chronic disease progression than house sparrows with salmonellosis.

Qualitative measures of body condition determined that the majority of salmonellosis cases were in thin or emaciated condition; however, there was a significant difference between species with the proportion of house sparrows that died of the disease in normal body condition being significantly greater than that for greenfinches. This finding supports a more rapid disease progression in the house sparrow than the greenfinch. Comparison of the quantitative variables confirmed that birds with salmonellosis were in significantly poorer body condition than the control group for each of the species, an observation in common with those of other authors'; however direct comparison between the greenfinch and house sparrow was not possible with this dataset.

The results of this study suggest that progression of salmonellosis in house sparrows is limited compared with greenfinches. The most likely explanation for this finding is that house sparrows die earlier during the acute course of the disease although the reason for this remains unclear. It is interesting to note that acute disease progression in house sparrows was also reported in New Zealand, although this was during a period of epidemic mortality following the emergence of *S.* Typhimurium DT160 as a novel phage type in the country (Alley et al., 2002).

Species-specific factors, at a genetic or immunological level, may alter the pathogenesis and progression of salmonellosis in passerines. Greenfinches appear more able to localise foci of infection versus more rapid dissemination to fatal septicaemia in the house sparrow. Greenfinches, therefore, may survive longer allowing the disease to progress to a more chronic state before death occurs. If this is the case, then greenfinches may be more likely to propagate the disease than other species such as the house sparrow. Pennycott et al. (1998a) proposed that greenfinches may be acting as a source of *Salmonella* infection for themselves and other birds at feeder sites. These results also support the implication that greenfinches may act as a reservoir species for *S.* Typhimurium infection (DT40 and DT56(v)) in wild birds.

Further work is needed to fully understand the epidemiology of *Salmonella* spp. infection and salmonellosis in garden bird populations. Evaluation of disease progression in other passerine species would be useful in determining the significance of greenfinches in the epidemiology of the infection. Histological assessment of other organs, particularly the spleen, would also enhance our understanding of the pathogenesis of passerine salmonellosis. Studies on *Salmonella enterica* infection in chickens suggest that the degree of faecal shedding may relate to a host's genetic background. Moreover, it has been proposed that certain *Salmonella* sp. may be able to modulate host immunity (Wigley et al., 2001; Barrow et al., 2004). In light of these findings it would be useful to know whether there is a specific difference in host immune response and faecal shedding between greenfinches and other species. Further

sampling of live birds may also help determine the prevalence of *Salmonella* spp. asymptomatic carriage in garden bird populations (Grant et al., 2007). *In vitro* salmonella persistence studies using greenfinch and house sparrow macrophages and *in vivo* experimental infection studies may be required to fully evaluate interspecific variation in salmonellosis.

Greenfinches and house sparrows are the passerine species most frequently affected by salmonellosis in Great Britain; these species share may behavioural and social habits, for example gregarious feeding and a granivorous diet. Garden bird feeding sites represent the most likely place where these 2 species regularly come in to close and sustained contact in high proximity. This study provides the first evidence to support interspecific variation between salmonellosis in passerine species. If the greenfinch acts as the species most frequently affected by the condition in which chronic disease progression occurs, it may also be the most likely source of infection to sympatric passerine species during infection or as an asymptomatic carrier. With this in mind, species mixing at feeding stations may play a role in the epidemiology of the infection.

CHAPTER 6: TRICHOMONOSIS LEADS TO RAPID POPULATION DECLINES OF COMMON BRITISH BIRDS

6.1 INTRODUCTION

Emerging infectious diseases (EIDs) are increasingly cited as threats to wildlife, livestock and humans alike (Daszak et al., 2000) and can be a major threat to geographically isolated or critically endangered wild bird populations (Bunbury et al., 2008). Parasites are integral components of healthy ecosystems, but while impacts on individuals are well recognised (e.g. Sheldon et al., 1996; Reed et al., 2008) consequences at the population level are poorly understood. Assessing the population impacts of disease, particularly those caused by emerging pathogens, within wildlife populations is problematic because little is known of their background complement of parasites and because the detection and diagnosis of disease in most wildlife species is challenging. Also, there usually is a paucity of host population data before and after disease emergence. Consequently, documented effects on the dynamics of common or widespread avian populations due to infectious disease are rare (van Riper et al., 1986; Hochachka et al., 2000; LaDeau et al., 2007). In this chapter the results of large-scale surveillance schemes for wild bird mortality are combined in order to quantify the incidence of an emerging disease in 3 widespread passerine bird species and its population impacts.

Trichomonas gallinae is a common protozoan parasite of pigeons (Columbiformes) which principally infects the upper alimentary tract where it can cause the disease, necrotic ingluvitis (Forrester et al., 2008). Epizootic mortality in columbiform species has been previously reported (Forrester et al., 2008) and the parasite infrequently affects other avian taxa such as birds of prey and songbirds (NWHC 2002; Anderson et al., 2009). Trichomonosis has been postulated to be a factor contributing to the extinction of the passenger pigeon (*Ectopistes migratorius*) (Stabler 1954) and has been shown to be a significant cause of nestling mortality in the island-endemic pink pigeon (*Nesoenas mayeri*) (Bunbury et al., 2008) and in the Iberian Peninsula population of the Bonelli's

eagle (*Hieraaetus fasciatus*) (Höfle et al., 2000; Real et al., 2000). Both of these small populations are considered to be endangered and, for both, mortality due to trichomonosis has been highlighted as a conservation concern.

Opportunistic surveillance of bird deaths in Great Britain since 2000 has shown a seasonal pattern in finch mortality driven primarily by salmonellosis incidents (Pennycott et al., 2006). In autumn 2005, the number of unsolicited submissions to the GBH*i* and Veterinary Laboratories Agency increased markedly and early investigations identified infection with a trichomonad parasite (Holmes et al., 2005; Pennycott et al., 2005a) as the probable cause. The geographic spread of these reports was uneven. In this chapter, advantage is taken of long-term monitoring of garden bird occurrence collected by a citizen science network to (i) quantify disease incidence in 3 species of common garden bird: greenfinch (*Carduelis chloris*), chaffinch (*Fringilla coelebs*) and dunnock (*Prunella modularis*); (ii) demonstrate a spatially contemporaneous decline in the occurrence of frequently affected bird species in gardens; and (iii) combine this with national monitoring of bird abundance by ornithologists to show that this decline was followed by significant reductions in regional breeding populations. Public reporting of wild bird carcasses has been utilised as a surveillance tool for West Nile virus and Usutu virus elsewhere (Eidson et al., 2001; Chvala et al., 2007). Volunteer networks were successfully instituted in North America to elicit reports of diseased birds in order to characterise the spread of mycoplasmal conjunctivitis in the house finch (*Carpodacus mexicanus*) (Dhondt et al., 1998; Hosseini et al., 2006). However, this study appears to be the first time that quantitative monitoring of disease incidence and its population impact has been undertaken using established survey networks.

6.2 MATERIALS AND METHODS

Identification of the disease epidemic

Since 2000, opportunistic nationwide monitoring of the causes of garden bird mortality in Great Britain has been carried out by the Institute of Zoology, the Wildlife Veterinary Investigation Centre and the Scottish Agricultural Colleges. Since 2000, the Royal

Society for the Protection of Birds (RSPB) has logged all reports of garden bird mortality and morbidity that the organisation has received from the general public where the history suggested that disease may be the cause. In 2005, these organisations, together with the British Trust for Ornithology (BTO), the Department of Veterinary Pathology, University of Liverpool and the Universities Fund for Animal Welfare, established a coordinated surveillance network as part of the Garden Bird Health *initiative* (GBH*i*) (Chapter 3). The GBH*i* was established before the emergence of trichomonosis in British finches was identified.

The GBH*i* surveillance of garden bird morbidity and mortality comprised opportunistic reports obtained from the general public and weekly reports from participants in a systematic reporting network. As opportunistic reports are vulnerable to temporal or spatial observer biases, for example following regional media reports, data from the systematic surveillance network was used to quantify disease incidence in an unbiased manner. This network utilised the BTO's Garden BirdWatch (GBW) volunteer network of approximately 15,000 households throughout Great Britain (Cannon et al., 2005). A random sample of 1,100 volunteers (stratified by the number of participants in each recording region) were approached with a view to recording additional information on bird morbidity and mortality in their gardens. Of those GBW participants approached, 751 participated in the GBH*i* project in 2006 (Chapter 3).

Identification of disease organism

Post mortem examinations (PMEs) using a standardised protocol were performed on carcasses submitted from a subset of reported garden bird mortality incidents (Chapter 4). Birds thought to have died as a result of 'Predation', 'Other trauma' or 'Infectious disease' were examined; the selection criteria did not specifically or solely target finch species or suspected cases of trichomonosis, but rather aimed to achieve a representative cross-section of species and aetiologies. In 2005 and 2006, a combined total of 995 garden birds of 42 species were examined, of which Fringillidae species accounted for 64% of submissions.

Fresh carcasses were submitted by post or hand-delivered and were refrigerated at 4 °C and examined fresh within 48 hours of submission where possible, or were frozen at -20 °C on submission and examined at a later date. Each submitted carcass was assigned a unique PME reference code and the species, age, sex and body weight were recorded. Systematic external and internal examinations of body systems were performed and any gross lesions described (Chapter 4). Where indicated, and when the degree of carcass decomposition permitted, samples were taken for microbiological, parasitological and histopathological investigations (Chapter 4). In addition, lesions (c. 5 mm^3) from cases with necrotic ingluvitis were incubated at 30 °C in Trichomonas Media No. 2. (Oxoid, UK) and screened for motile trichomonads at 24, 48, 72 hrs and 5 days.

A combination of morphological and molecular techniques was used to confirm the protozoan parasite species identification. Giemsa-stained preparations of trichomonad cultures were examined using light microscopy to assess parasite morphology. These were prepared by placing a drop of active trichomonas culture onto a standard glass microscope slide; this was then air dried, alcohol-fixed and stained using routine methods. Transmission and scanning electron microscopy was performed on trichomonad cultures fixed in 2.5% buffered gluteraldehyde and post-fixed in 1% osmium tetroxide (VWR, UK) at the University College Medical School, Royal Free Campus, using Philips 201 and 501 microscopes.

DNA was extracted from frozen/thawed necrotic ingluvitis lesions collected from finches, and from trichomonad cultures, using the Biosprint 15 DNA Blood Kit (Qiagen, UK) for purification of DNA from tissue according to the manufacturer's instructions. PCR was used to amplify the ITS1/5.8S/ITS2 ribosomal region using published TFR1 and TFR2 primers (Gaspar da Silva et al., 2007) with an adapted protocol. Briefly, PCR reactions were run with 3 µL of 10X PCR buffer (Qiagen, UK), 3 µL of 25mM $MgCl_2$ (Qiagen, UK), 0.5 µL of 5 U/µL HotStar Taq Plus DNA Polymerase (Qiagen, UK), 2 µL template DNA, 0.4 µL of 100mM dNTP mix (Bioline, UK), 3 µL of 10 µM forward and reverse primer and molecular grade water to complete the 50 µL per reaction. Oligonucleotide primers were supplied by Operon Biotechnologies (Germany). After an

initial 15 min denaturation at 94 °C, 35 cycles of 94 °C for 1 min, 65 °C for 30 sec and 72 °C for 1 min were carried out, followed by a 5 min extension at 72 °C using a thermal cycler (Tec-571, Techne, UK). Each PCR run contained a negative control of water and a positive control of purified trichomonad DNA obtained from parasites cultured from an affected greenfinch found dead as part of this study.

The PCR products, consisting of a clear single band, were visualised under UV light after ethidium bromide staining of a 1% agarose gel and the expected product size (c. 400 bps) was confirmed using Ready-Load 100bp DNA ladder (Invitrogen, UK). PCR products were purified using the QIAquick PCR purification kit (Qiagen, UK) and submitted for sequencing at the John Innes Genome Laboratory, UK, using the Applied Biosystems 3730xl with POP7 polymer and the TFR1 and TFR2 primers. Chromatograph profiles were inspected using Chromas 2 software (www.synthesisgene.com). Sequences from the forward TFR1 primer and the reverse complement of the TFR2 primer PCR product were aligned in both directions for each sample using MEGA 4.1 software and ClustalW (www.megasoftware.net). Sequences were compared with available gene sequences within the National Centre for Biotechnology Information (NCBI) Genbank using the Basic Local Alignment Search Tool (BLAST) search function to determine species identification within the Trichomonadidae.

Molecular detection of trichomonas infection

A nested PCR protocol was designed to increase the sensitivity of detection of trichomonad parasite DNA in template DNA extracted from lesions sampled post mortem and to provide a diagnostic tool for cases where autolysed carcass condition precluded *Trichomonas sp.* culture. This nested PCR targeted a fragment of the coding trichomonad small subunit rRNA (SSU) gene, synonymous with the 18S rRNA gene of eukaryotes as described by Gerhold et al. (2008) and mistakenly referred to as the 16S rRNA gene by Cepicka et al. (2005) (the latter term typically used to describe the SSU of prokaryotes). Trichomonad SSU rRNA primers (forward - TACTTGGTTGATCCTGCC and reverse - TCACCTACCGTTACCTTG) from

(Cepicka et al., 2005) were used for the first reaction. The PCR product nucleotide sequence was obtained from a pure trichomonad culture obtained from an affected greenfinch. Nested primers, TN3 forward (ATAGGACTGCAAAGCCGAGA) and TN4 reverse (TGATTTCACCGAGTCATCCA), were then designed using Prime3 online software (Rozen et al., 2000). Primers were supplied by Eurofins MWG Operon (UK).

The first stage used 2 µL of 10X PCR buffer (Qiagen, UK), 0.1 µL of 5 U/µL HotStar Taq Plus DNA Polymerase (Qiagen, UK), 2 µL template DNA, 0.4 µL of 10mM each dNTP mix (Qiagen, UK), 0.4 µL of 100 µM forward and reverse primer and molecular grade water to complete the 20 µL reaction. After an initial 5 min denaturation at 94 °C, 40 cycles of 94 °C for 1 min, 55 °C for 1 min and 72°C for 2 mins were carried out, followed by a 5 min extension at 72 °C using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, UK).

The reaction mixture for the second amplification was the same as for the first, except for the use of the nested primers. The amplification comprised 19 µl of mix and 1 µl of PCR product template from the first amplification round. After an initial 5 min denaturation at 94 °C, 35 cycles of 94 °C for 45 sec, 50 °C for 45 sec and 72 °C for 45 sec were carried out, followed by a 7 min extension at 72°C. Each amplification contained a negative control, consisting of water and a positive control of purified DNA obtained from cultured trichomonad parasites from a greenfinch. Amplified PCR products were visualised under UV light after ethidium bromide staining of a 3% agarose gel and the expected product size (c. 200 bps) was confirmed using Easy Ladder I (Bioline, UK). PCR products were submitted for sequencing at Cogenics (UK) using the ABI 3730 xl platform with the TN3 forward and TN4 reverse primers. Chromatograph profiles were inspected using Chromas 2 software. The sequence from the forward TN3 primer and the reverse complement of the TN4 primer PCR product were aligned in both directions for each sample using MEGA 4.1 software and ClustalW. Sequences were compared with available gene sequences within NCBI Genbank using the BLAST search function.

Reliable discrimination between necrotic ingluvitis due to salmonellosis or trichomonosis in finches is not possible based on gross examination alone. In order to evaluate whether the nested PCR cross-reacted non-specifically with *Salmonella* Typhimurium definitive type (DT)40 and DT56(v), microbiologically confirmed lesions from 56 greenfinches and 6 chaffinches with salmonellosis (Chapter 4) were screened. The majority of cases were negative on nested PCR (54/62 cases) and all cases examined during the study period 1st April 2006 to 30th September 2006 were negative. This indicates that 8 birds might have had concurrent infection with a *Trichomonas* sp. and *Salmonella* sp.. Carcass condition precluded parasite culture in all but 1 of these cases, but this case yielded trichomonad parasites confirming the existence of dual infection. These findings suggest that the nested PCR does not cross react non-specifically with *Salmonella* Typhimurium DT40 and DT56(v) and can be used within the case definition for the diagnosis of trichomonosis in finches.

Case definition

Cases of trichomonosis were diagnosed on the basis of the presence of necrotic ingluvitis lesions in combination with culture of motile trichomonads and/ or nested PCR amplification. Salmonellosis also causes necrotic ingluvitis and this was diagnosed by lesions being positive for a *Salmonella* sp. on culture. No cases of dual infection were identified during the study period, 1st April 2006 to 30th September 2006.

Geographical and temporal distribution of the epidemic

GBW participants (c. 9000) recorded the presence of a range of bird species encountered each week throughout the year; a subset (c. 30%) submitted actual counts of each species seen using an online recording form. Almost all the participants provided food of some kind for garden birds (the range of food provided was also recorded on a weekly basis) and feeding stations were generally the focal point of the study areas. The subset of GBW participants that took part in the GBH*i* surveyed all or part of their garden systematically in a consistent manner each week to record the number, and suspected cause, of dead or sick birds found. The clinical signs of ill health in birds affected by

trichomonosis typically included non-specific malaise, although dysphagia was noted in a large proportion of reported incidents.

In order to evaluate the geographical distribution of the trichomonosis epidemic, data from the opportunistic and systematic reporting schemes were examined for the period between 1st April and 30th September 2006. This interval was selected to minimise the likelihood of confounding these data with mortality due to salmonellosis, outbreaks of which occur during the winter months and which also result in non-specific signs of malaise in finches. Previous studies of salmonellosis in Great Britain (Pennycott et al., 2002), continental Europe (Refsum et al., 2002), and North America (Hall et al., 2008), and this study's findings in Chapter 2 & 4, have shown the disease to be seasonal, occurring almost exclusively within the period 1st October to 31st March across years.

Incident definition

As resources and logistics did not allow all dead birds found to be submitted for PME, specific criteria were established to determine if a mortality incident should be classified as likely being due to trichomonosis. For the purposes of this analysis, a suspected trichomonosis incident was defined, within the 6 month period of the study, as mortality included 2 or more dead finches (greenfinch or chaffinch), 1 or more sick finch(es) with typical signs of disease, or if trichomonosis was confirmed post mortem. The total number of trichomonosis incidents reported in each county per thousand households (according to the 2001 UK National Census (ONS 2005)) was calculated to give a measure of incidence for the opportunistic reports.

Changes in bird abundance

Each week, GBW participants recorded the presence of each bird species in their gardens and, optionally, the peak number of birds counted each week (Cannon et al., 2005). Analysis was based on the 912 gardens for which data were submitted in every week from January 2005 to June 2007 in England. Abundance was modelled as the proportion of gardens in which the species was present ('reporting rate'). Reporting rates were modelled using generalised additive or linear mixed models, fitted using the gamm

function in package *mgcv* for R 2.6.0 (Wood 2006; R Development Core Team 2007). This model was developed by Rob Robinson at the BTO who led the analyses to assess population level impact, in collaboration with the author, presented in this chapter.

To exclude the possibility that changes in other environmental factors, such as climate, between years might have caused changes in greenfinch numbers, the abundance of chaffinches, which have a similar body size and ecology to the greenfinch and were the second most frequently affected species in which trichomonosis was recorded, and dunnocks, which also feed around garden feeders (Snow et al., 1998) and in which trichomonosis was rarely recorded (only 3 cases diagnosed in 2005 and 2006 combined), were also modelled. Both species show similar patterns of spatial and temporal abundance in gardens to the greenfinch (Cannon et al., 2005). If trichomonosis was responsible for changes in numbers, chaffinch would be predicted to show an identifiable, but less marked, response and dunnock should show little response; if other factors, such as climatic factors or a change in resource availability, were involved then the response among the 3 species might be similar.

Abundance of birds in gardens varies seasonally in a non-linear fashion, with peak numbers occurring in late winter or spring and lowest counts in late summer/autumn, when many birds are moulting (Cannon et al., 2005; Chamberlain et al., 2005). To model this variation the BTO fitted a generalised additive model to the reporting rate as a function of week number (1-7 Jan = 1 to 25-31 Dec = 52) in the form of a thin-plate regression spline using gamm with a binomial error structure and logit link function (Wood 2006). The default level of smoothing was used, since increasing the potential for smoothing (by changing the basis, k) did not alter the results materially. To account for variation among gardens in the number of birds present and observer ability, garden identifier was included as a random-effect term. As weekly abundance values are likely to be serially correlated an auto-regressive correlation structure (with lag=1 with weeks numbered consecutively through the entire period) was also incorporated; the degree of auto-correlation was assumed to be the same for each garden. Counts were assumed to be independent among gardens as the distance between gardens was generally much

greater than the ambit of individual foraging flocks and it is unlikely the provisioning behaviour in any one sample garden will influence the behaviour in neighbouring sample gardens. Furthermore, there was relatively little spatial variation in reporting rates with these species being reported in most (>70%) gardens throughout the area of interest.

To quantify impacts on the breeding season following the epidemic of trichomonosis in autumn 2006, the BTO constructed a model to compare reporting rate at the start of the 2007 breeding season (weeks 13-21, 26 March – 28 May) with the average reporting rate for the same period in the previous 2 years (which, as far as could be determined, were typical). The analysis was restricted to the previous 2 years to avoid potential confounding effects of any long-term trends in bird numbers and reporting rates. Since a relatively short time-span (within a year) was considered in this study, it was not necessary to fit a smoothed term of week; rather reporting rate was modelled as a function of garden size, week (and its square to account for any non-linearity) and a two-level dummy variable, year, with weeks in 2005/06 having year=0 and those in 2007 year=1. The estimate of this latter term then gives the change in abundance in 2007 relative to that in the previous 2 years. As before, garden identifier was included as a random effect and fitted an auto-correlated binomial error structure.

Mortality due to trichomonosis varied spatially throughout the country, so 3 regions, representing areas of High, Intermediate and Low incidence of trichomonosis, were defined based on the results of opportunistic sampling. Analyses were restricted to England south of a line from the Mersey to the Humber (approx. 53° 30'N) as it is in this region that gardens participating in GBW are most representative of the landscape as a whole (for example upland areas, where there tend to be few GBW sites, are relatively limited in extent). Greenfinch, chaffinch and dunnock populations occur widely across this region being observed in 70-80% of gardens in the GBW scheme (BTO 2010). For convenience, these 3 regions were defined in terms of administrative county boundaries with areas of High (Cheshire, Derbyshire, Gloucestershire, Herefordshire, Leicestershire, Shropshire, Staffordshire, Warwickshire and the West Midlands),

Intermediate (Bedfordshire, Buckinghamshire Cambridgeshire, Lincolnshire, Northamptonshire, Nottinghamshire, Oxfordshire and Wiltshire) and Low (Berkshire, Essex, Hampshire, Hertfordshire, London, Kent, Norfolk, Suffolk, Surrey and Sussex) incidence of trichomonosis. To test for differences in the change in abundance in spring 2007 among areas, an interaction term was included between area and the dummy year variable described above.

Numbers of birds present across Great Britain during the breeding season are monitored using line-transect counts in a sample of c. 3,000 randomly selected 1x1 km squares by the Breeding Bird Survey (BBS[1]) (Newson et al., 2008). BBS transects are undertaken in all habitats, rather than being restricted to gardens as with the GBW scheme. An index of relative abundance based on a generalized linear Poisson model with categorical site and year fixed-effects is produced annually; indices were obtained for the 3 county groupings to measure the relative change in breeding population between 2006 and 2007 in each region.

6.3 RESULTS

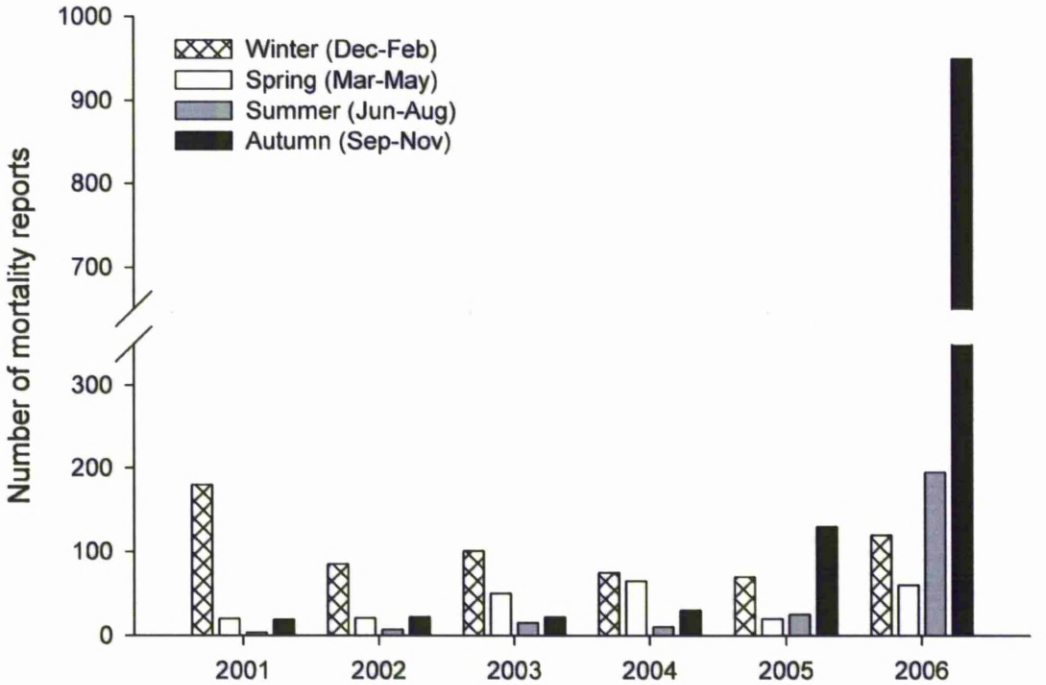
Identification of the disease epidemic

Opportunistic monitoring of garden bird mortality by the RSPB between 2001 and 2004 showed an annual seasonal peak in mid-winter (Dec/Jan), with 37-76% of reports per annum occurring in these 2 months (Figure 6.1, K. Peck, *unpublished data*); PMEs indicated that this seasonal peak was largely due to salmonellosis in Fringillidae and Passeridae species (Pennycott et al., 2006; Kirkwood, Cunningham and Simpson,

[1] The Breeding Bird Survey (BBS) monitors terrestrial birds throughout the UK to provide information that underpins the conservation of species and habitats. The BBS is organised by the BTO, and jointly funded by BTO, the Joint Nature Conservation Committee (JNCC, on behalf of the Council for Nature Conservation and the Countryside, the Countryside Council for Wales, Natural England and Scottish Natural Heritage) and the RSPB.

unpublished data). Between January 1st 2000 and December 31st 2004, 750 garden birds, including 264 greenfinches, were examined post mortem from across Great Britain with most finch deaths due to salmonellosis and no cases of trichomonosis in finches confirmed.

Figure 6.1: Seasonal incidence of opportunistic reports of garden bird morbidity and mortality collected by the RSPB (K. Peck *unpublished data*) where the history indicated that infectious disease may be involved, 2001-2006. Note break in axis indicating an unprecedented level of reporting in autumn 2006.



Following the index case of trichomonosis in a British finch in April 2005, small numbers of finch mortality incidents were reported throughout 2005, unusually peaking during September to November (Pennycott et al., 2005a). In summer 2006, the number of finch, particularly greenfinch, mortality reports increased dramatically with a total of 1054 suspected trichomonosis incidents recorded (involving c. 6300 dead greenfinch and chaffinch (combined), (according to the incident definition)), between 1st April 2006 and 30th September 2006. This comprised 55% of all reported 1912 incidents of garden bird

morbidity and mortality during this period and compares with 84 incidents of suspected trichomonosis for the same time period in 2005 and sporadic cases (none of which involved finches) in previous years since 2000. These reports from the public were unsolicited, not in response to a direct appeal and occurred prior to media coverage of the EID. Sick and dead birds at affected sites were typically observed in close vicinity to garden bird feeding stations and exhibited non-specific signs of malaise, for example lethargy and fluffed-up plumage, frequently in combination with dysphagia (Figure 6.2).

Figure 6.2: Sick greenfinch exhibiting clinical signs typical of trichomonosis (a) fluffed-up plumage and lethargy, (b) food staining around the beak and open-mouth breathing.

(a)

(b)

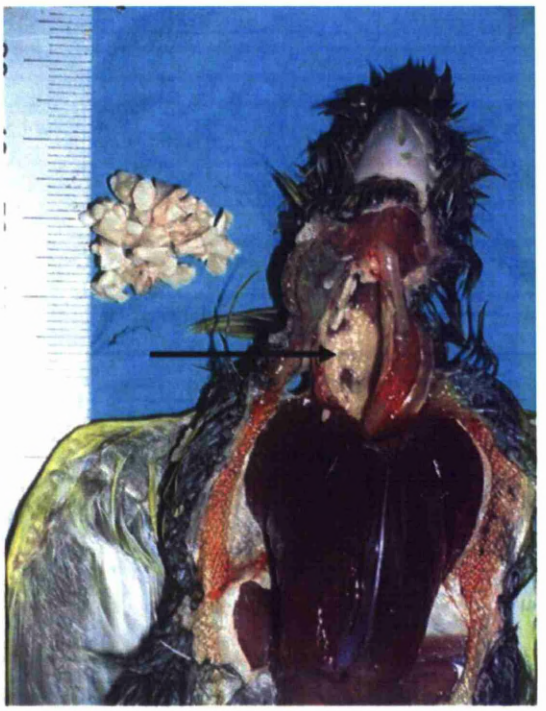
Identification of the disease organism

Necrotic ingluvitis, typically extending through the full thickness of the oesophageal wall and often involving adjacent connective tissue, was diagnosed through PME (Figure 6.3) and confirmed as trichomonosis, according to the case definition, in 70 of 125 greenfinches, and in 18 of 76 chaffinches examined between 1st April 2006 and 30th September 2006. These diagnoses were reached on the basis of *T. gallinae* culture alone in 17 birds, nested PCR amplification alone in 58 birds, and a combination of parasite culture and nested PCR in 13 birds. All confirmed trichomonosis cases were negative for *Salmonella* sp. on culture. In addition, 20 greenfinches and 29 chaffinches were suspected to have died of trichomonosis as these birds had necrotic ingluvitis which was negative for *Salmonella* sp. Trichomonosis in these cases, however, was not confirmed using nested PCR or culture.

Of 241 finch carcasses examined during the period 1st April 2006 to 30th September 2006, 179 were diagnosed to have died as a result of infectious disease. Of these 179 birds, trichomonosis accounted for 144 (80%) of the deaths, *E. coli* serotype O86 (the second most common infectious cause of death) was diagnosed in 24 (13%) birds, while salmonellosis was confirmed in only 4 (2%) birds.

Figure 6.3: Necrotic ingluvitis in a greenfinch. a) Necrotic ingluvitis lesions (arrow) with a characteristic yellow caseous appearance in a greenfinch caused by *T. gallinae* infection. Recently ingested sunflower seed hearts, removed from the ingluvium, are shown to the left of the carcass. (b) Necrotic ingluvitis lesions and empty gizzard with black-stained tarry contents.

(a)



(b)

Photo credit Julian Chantrey

Fringillidae species accounted for 84% (292/347) of trichomonosis cases diagnosed and columbidae species accounted for 11% (37/347) of cases in 2005 and 2006 (Table 6.1).

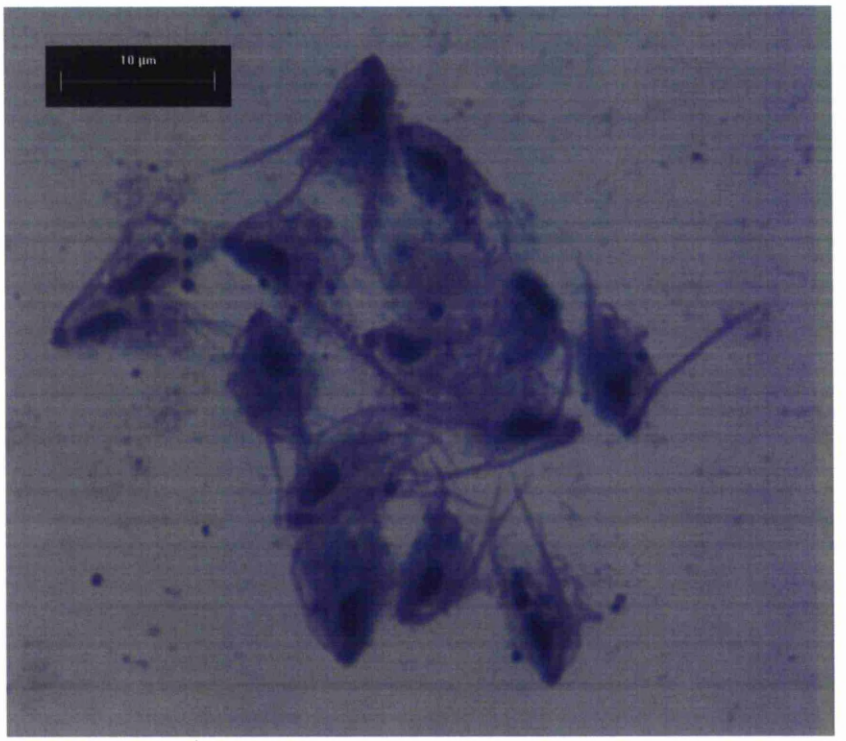
Table 6.1: Trichomonosis cases (suspected and confirmed) in 2005 and 2006

Family/ Species	**Number of cases**
Columbidae	
Collared dove *Streptopelia decaocto*	20
Woodpigeon *Columba palumbus*	17
Fringillidae	
Brambling *Fringilla montifringilla*	2
Bullfinch *Pyrrhula pyrrhula*	5
Chaffinch *Fringilla coelebs*	106
Goldfinch *Carduelis carduelis*	4
Greenfinch *Carduelis chloris*	173
Siskin *Carduelis spinus*	2
Yellowhammer *Emberiza citrinella*	4
Passeridae	
House sparrow *Passer domesticus*	9
Prunellidae	
Dunnock *Prunella modularis*	3
Paridae	
Great tit *Parus major*	2
Total	347

Concurrent soiling of the beak and facial plumage with food and saliva was frequently present in affected finches and such birds were typically thin or emaciated. Histopathological examination of the crop confirmed focally extensive moderate to severe mucosal ulceration and submucosal necrosis with infiltration by moderate numbers of degenerate and viable heterophils, lymphocytes and macrophages. Superficially, there often was a layer of necrotic crop epithelial tissue within which groups of 10-20 μm diameter round cells (consistent with protozoal organisms) and numerous clusters of mixed bacterial colonies were seen. Autolysis of the alimentary tract precluded meaningful histological examination in many cases; consequently histopathology was not used as a routine diagnostic test for confirmation of trichomonosis.

Giemsa-stained parasite culture preparations revealed a variable morphology (body dimensions range 8-11 x 4-5 μm) typical of a trichomonad parasite with a single nucleus and axostyle, anterior flagella and an undulating membrane (Figure 6.4).

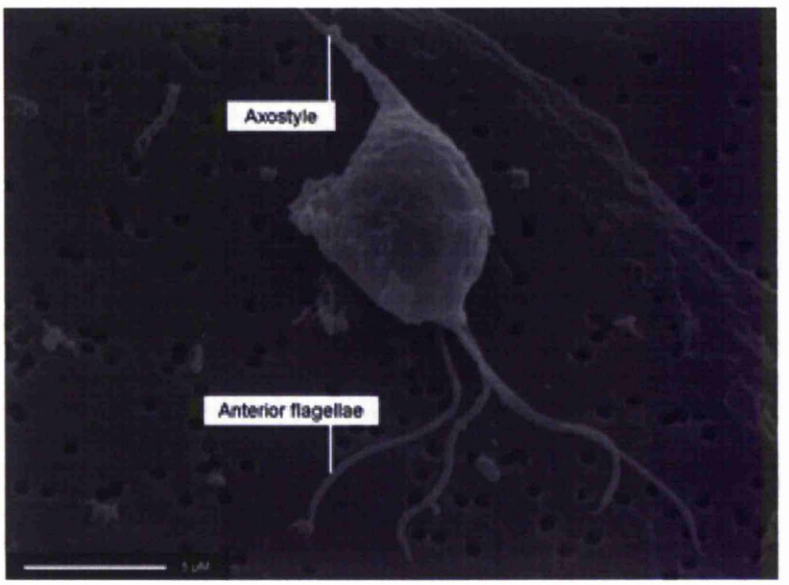
Figure 6.4: Giemsa preparation showing trichomonad parasite morphology.



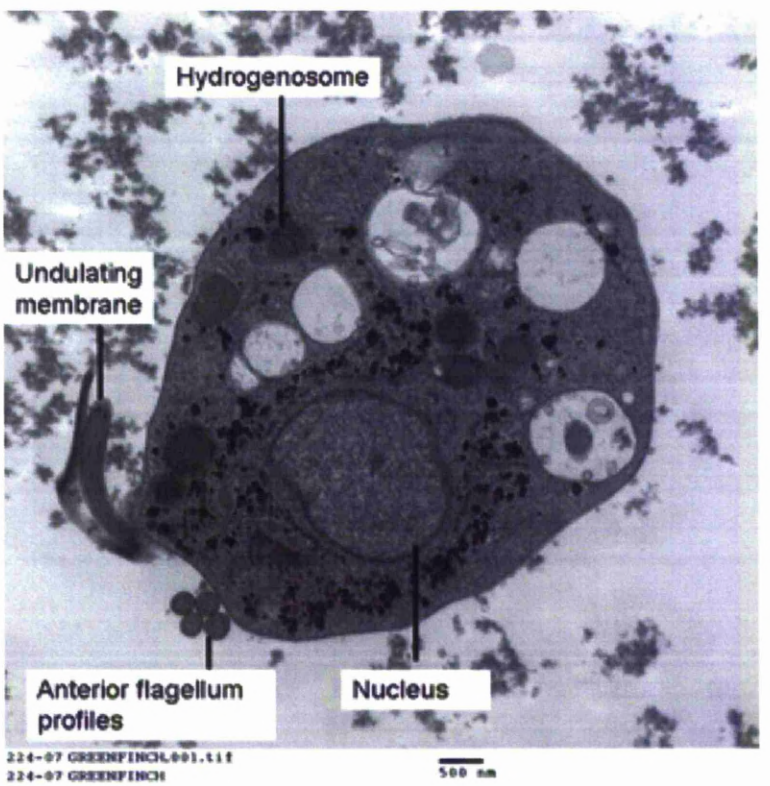
Scanning and transmission electron microscopy (Figure 6.5) confirmed the presence of a parasite with plastic pyriform morphology and 4 anterior flagella that typically exited the body together in pairs. A prominent undulating membrane, with no free posterior trailing flagellum, was present.

Figure 6.5: Morphology of the greenfinch trichomonad parasite. (a) Scanning electron micrograph. Arrows indicate anterior flagella and axostyle (b) Transmission electron micrograph showing cell ultra-structure.

(a)



(b)



Amplification of the ITS1/5.8S/ITS2 ribosomal region was performed on DNA extracted from oesophageal lesions from 9 greenfinches and 9 chaffinches (submitted from 18 disparate sites across 13 counties covering England, Wales and Scotland) that died of trichomonosis in 2005 or 2006. An identical consensus sequence of 214 nucleotides was identified for all PCR products (Figure 6.6a, Genbank accession numbers GQ150752 and GQ150753) from the finch samples examined. NCBI BLAST search identified that the consensus finch sequence matched 4 Genbank entries with 100% sequence homology and 100% query coverage, all of which were for *T. gallinae* (EU215369, EU290649, EF208019, AY349182). Thus the organism infecting the British finches was identified as *T. gallinae*.

Figure 6.6. Sequence data from British finch trichomonad samples (a) Nucleotide sequence (214 nucleotides) from amplification and sequencing of the ITS1/5.8S/ITS2 ribosomal region using TFR1 and TFR2 primers from (A) Consensus sequence from British finch (Genbank GQ150752 and GQ150753) trichomonad samples and (B) *Trichomonas gallinae* (Rivolta) Stabler (ATCC® Number 30230). (b) Consensus sequence from British finch trichomonad (149 nucleotides) from nested PCR with trichomonad SSU rRNA primers followed by TN3 and TN4 nested primers (Genbank GQ214405).

(a)

A1. TAACTTCATCAAAAAATCAAGTCTCTAAGCAACGGATGTCTTGGCTCCTC
B1. TAACTTCATCAAAAA-TCAAGTCTCTAAGCAACGGATGTCTTGGCTCCTC

A51. ACACGATGAAGAACGTGGCATAATGTGTTAAGTAACCGGAGTTGCAAACA
B51. ACACGATGAAGAACGTGGCATAATGTGTTAAGTAACCGGAGTTGCAAACA

A101. TCATGACAGGTTAATCTTTGAATGCAAATTGCGCTTACCCGGCTTCGGCC
B101. TCATGACAGGTTAATCTTTGAATGCAAATTGCGCTTACCCGACTTCGGTC

A.151 GAGGAGCATGCGTGTAACAGTACAACATAATTTATAATAATTCTTATTCT
B151. GAGGAGCATGCGTGTAACAGTACAACATAATTTATAATAATTCTTATTCT

A201. ACGCGAATAAGCAA
B201. ACGCGAATAAGCA

(b)

1. GGCCGCGCTACTCTTATAATCCCTAACGTAGTTGGGATTGACGTTTGTAA
51. TCAGCGTCATGAACCAGGAATCCCTTGTAAATGTGTGTCAACAACGCACG
101. TTGAATACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGAT

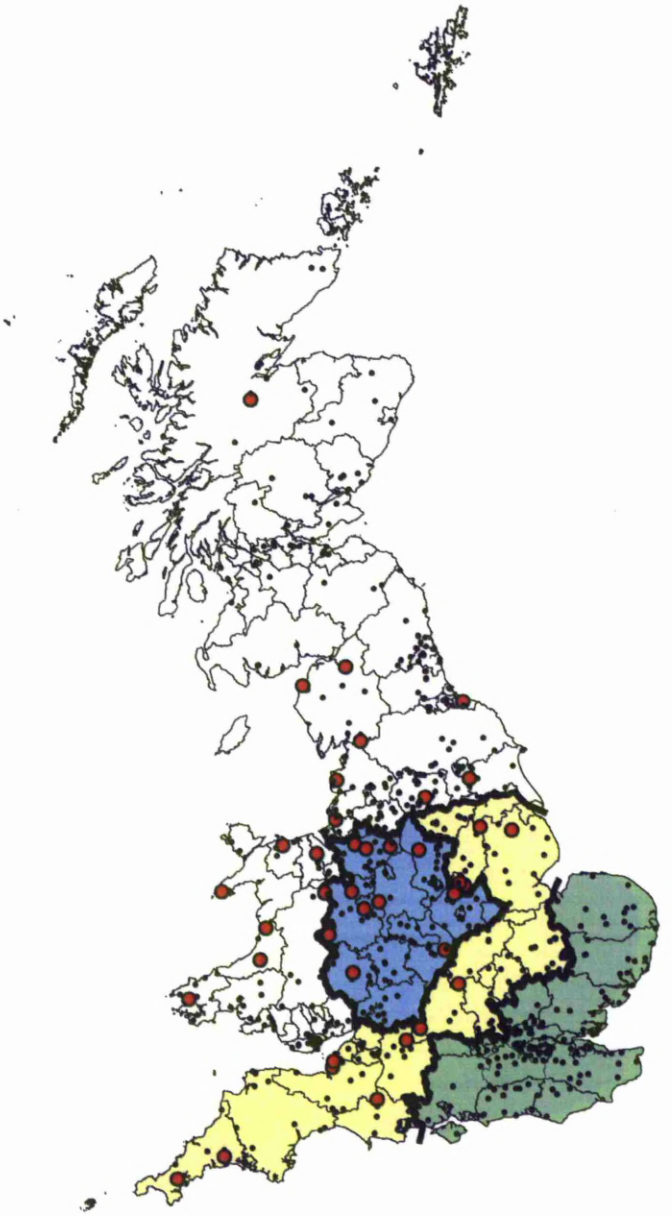
Sequencing of second stage products of a nested PCR for the detection of trichomonads from oesophageal lesion extracts from 7 greenfinches and 1 chaffinch examined in 2005

or 2006 with trichomonosis identified a consensus sequence of 149 nucleotides in all 8 cases (Figure 6.6b, Genbank accession no GQ214405). NCBI BLAST identified the consensus finch sequence as a match for 4 *T. gallinae* Genbank entries (EU215372.1, EU215373.1, EU215374.1 and EU215375.1) with 100% sequence homology and 100% query coverage and 5 *Trichomonas* sp. reports with 100% sequence homology and 97% query coverage. Multiple *Tetratrichomonas gallinarum* and *Tetratrichomonas* sp. reports had 98% or less sequence homology and 100% query coverage.

Geographical and temporal distribution of the epidemic

Rates of opportunistic reports of trichomonosis (identified according to the incident definition) varied greatly among counties, with rates in excess of 0.20 incidents per thousand households (ipth) found in Gloucestershire, Powys and Warwickshire. Opportunistic reports were used to define regions of High, Intermediate and Low disease incidence (Figure 6.7). Overall, the average reporting rate was 0.037 ipth but this varied markedly among the regions (aggregate average for the High region: 0.109 ipth; Intermediate region: 0.056 ipth; Low region: 0.003 ipth, X^2=650.5, df=2, P<0.001). As these rates may have been influenced by local publicity, incidence was quantified at systematically monitored garden sites throughout the study period. Trichomonosis (identified according to the same incident definition) was identified at 39 systematically monitored sites (5.2%) but the incidence varied spatially in a manner similar to the opportunistic reports, with 10.4% (n=115) of participants in the High region reporting an incident, 7.8% (n=154) in the Intermediate region and 0.0% (n=164) in the Low region (X^2=16.4, df=2, P<0.005).

Figure 6.7: Distribution of suspected finch trichomonosis incidents in 2006. Gardens reporting at least 1 suspected incident of finch trichomonosis (large red dots) and all other sites (small black dots) contributing to the systematic survey. The shaded regions delimit the 3 regions of High (blue), Intermediate (yellow) and Low (green) incidence of trichomonosis incidents in the 2006 epidemic used for the analyses.

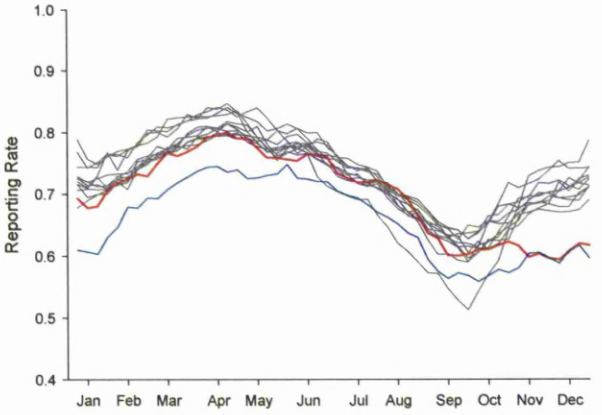


Changes in bird occurrence

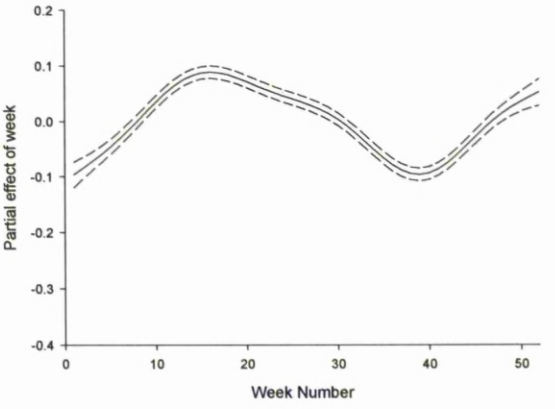
The weekly reporting rates of greenfinch occurrence in all gardens contributing to the BTO GBW survey (Cannon et al., 2005) show a seasonal pattern, with more gardens reporting birds in spring and fewer in the autumn (Figure 6.8a). There was a significant difference in seasonal pattern of occurrence between 2005 (which was very similar to previous years, Figure 6.8b) and 2006, with markedly fewer gardens reporting greenfinches from early August (week 32) onwards (Figure 6.8c, $F=24.23$, $P<0.0001$). Analysis of a subset of these sites which recorded counts of individual birds (rather than presence) showed a similar reduction in mean abundance in gardens reporting greenfinches in the latter half of 2006.

Figure 6.8: Seasonal variation in greenfinch occurrence in gardens (a) Reporting rate for greenfinch in all GBW gardens for the years 1996-2005 (grey lines), 2006 (red) and 2007 (blue). (b) Fitted seasonal pattern of mean peak greenfinch count in 912 GBW gardens with complete counts in 2005. (c) Difference in mean peak count throughout the year between 2005 and 2006 for greenfinch, dashed lines represent 95% confidence limits.

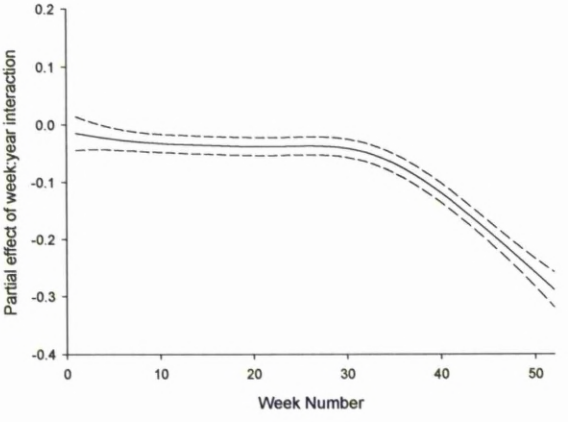
(a)



(b)



(c)



The reporting rate for greenfinches in the following spring (2007) was significantly reduced in the area of High trichomonosis-associated mortality (β = -1.32 ± 0.12, P<0.001), but less so in the region with Intermediate (β = -0.77 ± 0.15, P<0.001) or Low mortality levels (β = -0.53 ± 0.08, P<0.01) (Figure 6.9). Reductions in occurrence of chaffinch (β = -0.53 ± 0.12, P=0.02) and dunnock (β = -0.25 ± 0.12, P=0.04) in the region of High trichomonosis-associated mortality were lower, and there were no significant reductions in occurrence of either species in the regions of Intermediate or Low incidence.

Figure 6.9: Regional change in greenfinch occurrence in gardens in response to trichomonosis. Mean reporting rate from GBW of (a) greenfinch, (b) chaffinch and (c) dunnock in spring 2005/06 (filled bars) and 2007 (open bars) in areas of Low, Intermediate and High trichomonosis incidence (see Figure 6.7). Bars represent 95% confidence limits.

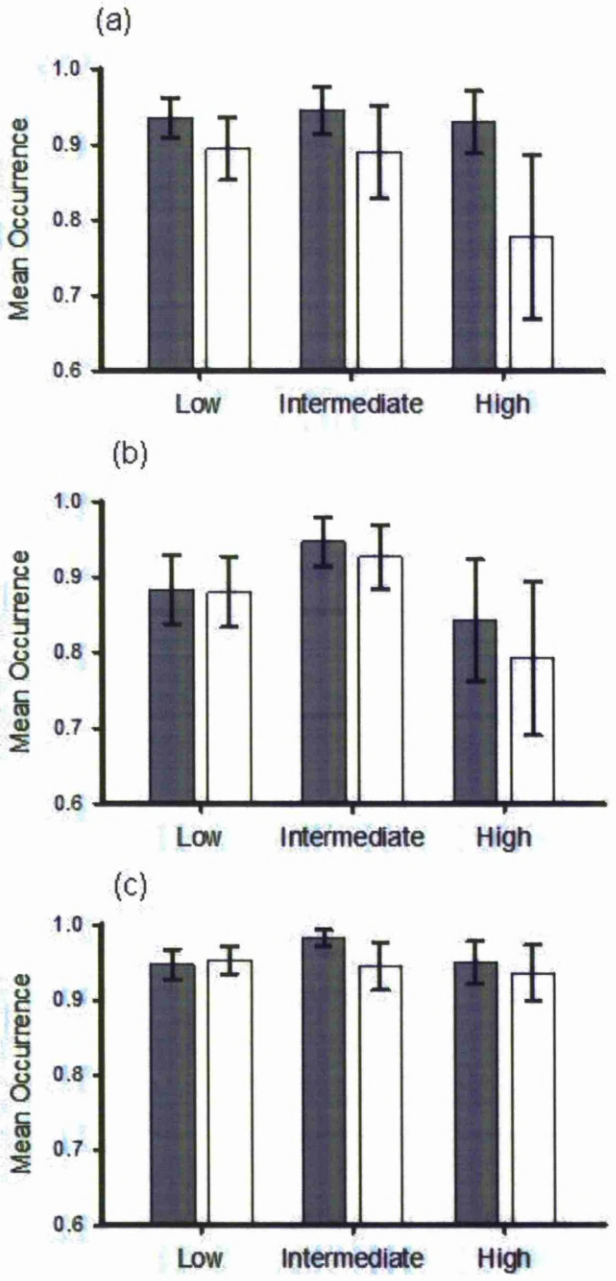
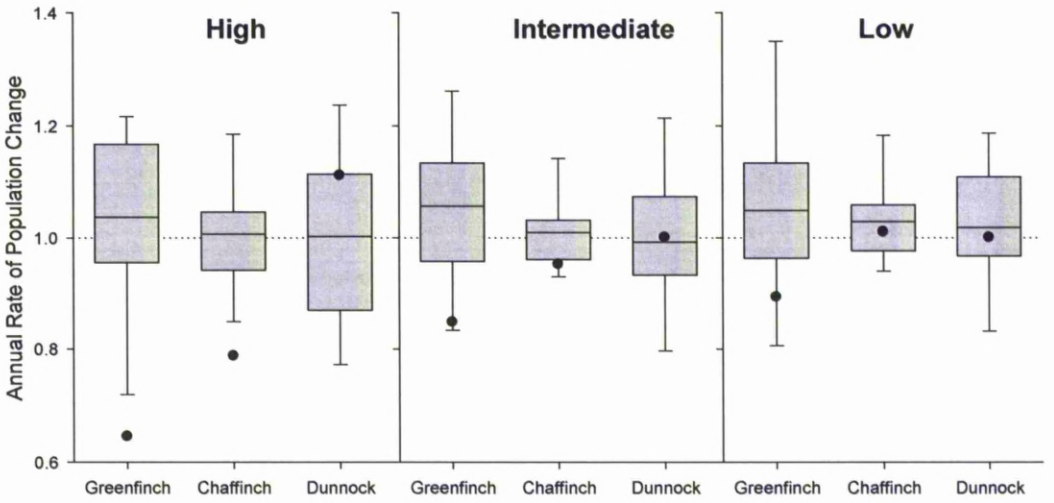


Table 6.2: Population change between 2006 and 2007 in areas of High, Intermediate and Low incidence of trichomonosis-associated mortality during autumn 2006 derived from the Breeding Bird Survey. The percentage change between the 2 years is given with approximate 95% confidence limits. The number (n) of monitored BBS sites included in each calculation is given.

	Low		Intermediate		High	
	n	% Change	n	% Change	n	% Change
Greenfinch	477	-10.9 (-17.3, -4.1)	433	-15.2 (-21.8, -8.1)	232	-35.5 (-42.3, -27.9)
Chaffinch	549	+0.6 (-3.7, +5.1)	526	-4.4 (-8.7, +0.2)	256	-21.3 (-25.7, -16.5)
Dunnock	496	-1.2 (-7.0, +7.3)	465	-0.3 (-7.7, +7.6)	286	+11.4 (+1.7, +22.1)

These observed reductions in occurrence were reflected in changes in the size of wider regional breeding populations obtained from the independently-derived BBS (Newson et al., 2008). The decline in relative abundance of greenfinches on BBS squares was significantly greater (35%) in the region of High trichomonosis-associated mortality than in either of the other 2 regions (Table 6.2). In accordance with the study predictions, this annual change was much more marked than any seen in the previous 10 years (Figure 6.10). Likewise, as predicted, the number of breeding chaffinches declined significantly (21%) in the High incidence region but not in the other 2 areas; abundance of breeding dunnocks did not decline in any region.

Figure 6.10: Annual rate of population change as measured by the BBS in areas of differing disease incidence. Boxes show mean and quartiles of annual changes and whiskers minimum and maximum annual change observed in the period 1994-2006; points, the population change recorded in 2007. Dotted line indicates no population change.



6.4 DISCUSSION

Trichomonosis was diagnosed as an emerging and widespread cause of death in British finches in 2005 and 2006. The gross and histopathological findings are consistent with upper alimentary tract lesions caused by *T. gallinae* infection in columbiform species (Perez-Mesa et al., 1961; Narcisi et al., 1991), although lesions in finches typically occur in the proximal oesophagus, rather than the pharyngeal region in affected pigeons and doves. Ultrastructural examination identified morphology consistent with *Trichomonas* sp. protists, including *T. gallinae* (Soulsby 1968; Bondurant et al., 1994; Benchimol 2004). Amplification and sequencing of the ITS1/5.8S/ITS2 ribosomal region using TFR1 and TFR2 primers yielded consensus sequence with 100% homology to published accounts for *T. gallinae*, confirming the parasite species identification (Kleina et al., 2004; Gaspar da Silva et al., 2007; Gerhold et al., 2008; Anderson et al., 2009).

Experimental assessment of the survival of *T. gallinae* in white-winged dove (*Zenaida asiatica*) carcasses found that most reliable diagnostic results were obtained on sampling within 8 hours of death and that parasite culture from *T. gallinae*-positive birds was successful in only 44% of carcasses sampled at 48 hours following death (Erwin et al., 2000). In the current study, parasite culture was found to be a useful technique for confirmation of the diagnosis in carcasses even in a mild state of autolysis. Due to delays in submission, however, negative culture results could not be used to exclude the diagnosis. Therefore, nested PCR provided a useful ancillary diagnostic tool in combination with post mortem and microbiological examinations within the case definition for the diagnosis of trichomonosis for carcasses in which the protozoan parasites were no longer viable. The specificity of the nested PCR for a range of species within the Trichomonadidae was not assessed in this study therefore this technique cannot currently be used in isolation as a diagnostic test for *T. gallinae*. However, use of the nested PCR appeared robust within the case definition for finch trichomonosis employed in this study.

Consideration of 2 independent data sets of bird mortality in Great Britain (i.e. (1) opportunistic surveillance based on unsolicited reports from members of the public and (2) systematic surveillance through the pre-existing GBW network) in combination enabled their respective benefits to be maximised. The large-scale, but *ad hoc*, sampling from the opportunistic survey indicated the onset of the epidemic and provided samples to determine its epidemiology. The systematic surveillance provided a quantitative measure of disease incidence which was relatively robust to, for example, increases in reporting frequency in response to media coverage.

The winter of 2006 was relatively mild (mean temperature anomaly in England for Nov-Mar +1.7°C (Met Office 2010)) so mortality levels would be expected to be low (Robinson et al., 2007). Also, the number of birds, especially seed-eaters, recorded in gardens would be expected to be lower than usual due to a reduced reliance on provisioning (Chamberlain et al., 2005). However, the observed reduction in the occurrence of greenfinches in gardens was not simply because birds were not coming

into gardens for food, since reporting continued to be low into the following spring (Figure 6.8a) and regional declines in greenfinch breeding populations were observed in an independent dataset (BBS) with broader coverage. The onset of the decline in greenfinch reporting rate was contemporaneous with the onset of the trichomonosis outbreak (Figure 6.8a) and the spatial pattern in regional population decline (Table 6.2) matched that of the disease occurrence (Figure 6.7). It was not possible to identify any other factor that could have caused such large-scale mortality. Furthermore there were no similarly large declines noted in any other garden bird species (Toms, *unpublished data*).

In Great Britain as a whole, the greenfinch breeding population increased steadily (by c. 60%) from the mid 1980s to 2006, but decreased (significantly) by 15% in 2007 compared with the previous year (Risely et al., 2008). It seems likely that this decline was driven, in large part, by the emergence of trichomonosis. Given that the population of greenfinches in Great Britain is in the order of 4 million (Newson et al., 2008), this represents mortality of a very large number of individuals, possibly in the order of half a million birds. This level of mortality due to an EID is unprecedented in British wild bird populations and is of international interest as, although other studies have demonstrated trichomonosis in non-columbiform species (Anderson et al., 2009), this is the first trichomonosis epidemic to show substantial negative impact on free-ranging populations of these species. Forrester et al. (2008) recently commented that the largest trichomonosis epidemic on record was estimated to involve mortality of 50,000 to 100,000 American mourning doves (*Zenaida macroura*) in the south-eastern states of U.S.A. between 1950 and 1951 (Haugen 1952; Haugen et al., 1952). Consequently, it is plausible that the epidemic mortality due to trichomonosis that occurred in Great British finches in 2006 represents the greatest absolute wild bird mortality attributed to the disease worldwide.

The current study also reinforces the value of large-scale, long-term, citizen science programmes for wildlife population monitoring and disease surveillance and of a multi-disciplinary approach to investigating the conservation significance of wildlife disease.

As the opportunistic reports from the public were unsolicited, rather than in response to a direct appeal such as that undertaken to monitor the spread of house finch mycoplasmosis in the U.S.A. (Dhondt et al., 1998), large-scale underreporting during the trichomonosis epidemic of 2006 is likely to have occurred. However reports from the systematic GBW network, where participants searched their garden in a systematic fashion should provide a more accurate estimate of disease incidence (with up to 10.4% of gardens affected) and this was temporo-spatially consistent with the observed declines in greenfinch and chaffinch populations.

Anderson et al. (2009) found a low prevalence of infection in house finches and hypothesised that endemic infection may only have been present in California since the introduction of rock doves (*Columbia livia*) and the increase in feeding of garden birds in backyards. Their study found a high case mortality rate in the house finch, with no evidence for asymptomatic carriage, and postulated that the apparent lack of carrier state may be due to only recent exposure to the parasite with no selective pressure to encourage host adaptation. Whilst the impact of the trichomonosis epidemic has been much more dramatic on British, than Californian, finch populations, this same explanation may account for the high levels of mortality and population declines of the greenfinch and chaffinch that have occurred in Great Britain. Gaspar da Silva et al. (2007) also postulated that the apparent greater susceptibility of the island endemic pink pigeon to infection with *T. gallinae*, as compared with sympatric introduced or exotic columbid species, may be due to the short period of exposure and opportunity for host adaptation to the parasite.

The origin of *T. gallinae* infection in finches is currently unknown but columbiform species are the most likely source (Chapter 8). Retrospective surveys using the nested PCR methodology on archived garden bird tissues, collected prior to 2005, could identify presence of the parasite in non-columbiform populations prior to the onset of epidemic finch mortality, although the likelihood of detecting a low prevalence from the relatively small number of archived carcasses would be small.

Although greenfinches appeared to be the species most frequently affected by *T. gallinae* infection in garden habitats, including the closely-related chaffinch, the reasons for this are not clear. The greenfinch, also, is one of the species most frequently affected by other infectious diseases that are commonly diagnosed in garden birds, such as salmonellosis and colibacillosis (Chapter 4). Mortality of other finch species due to trichomonosis in smaller numbers was also diagnosed. The gregarious and granivorous habits of finch species sharing food and water at feeding stations with high contact rates might facilitate pathogen spread. Trichomonosis, however, was confirmed only rarely in Paridae species, which also commonly flock to garden bird feeders, so feeding behaviour is unlikely to be the sole driver of greenfinch susceptibility. Investigations of wildlife species mortality which rely on the reporting of sick and dead birds by members of the public have an inherent risk of bias due to observer effort. Also, some species, such as large or brightly coloured ones, are more likely to be detected. Reporting by the participants in the systematic scheme provided a known level of observer effort which is likely to have reduced the magnitude of any detection bias (Chapter 3). Although the occurrence of trichomonosis in dead birds has been quantified in this study, the prevalence of *T. gallinae* infection in wild birds remains unknown. Prospective studies to screen multiple species of live birds for trichomonad parasites (Bunbury et al., 2005) would help address this knowledge gap. Experimental challenge studies in multiple species are required to definitively confirm the extent of interspecific variation in susceptibility to infection with, and to disease caused by, *T. gallinae*.

Land-use change and habitat degradation have led to an increased national focus on garden habitats as a useful refuge for British wildlife (Toms 2007). It has been estimated that 48% of gardens in Britain provide some form of artificial food for wildlife (Davies et al., 2009). A recent review of anthropogenic provisioning of wild birds in garden habitats by Robb et al. (2008) reported that the practice increases bird density at feeding stations and noted recent concern for pathogen exposure and transmission that might occur as a consequence. Garden bird feeding practice in Great Britain has altered over recent years with increased adoption of summer feeding and increased provision of

sunflower and niger seed which might have led to increased concentration of birds at feeders. *T. gallinae* can be transmitted through direct contact between birds, for example courtship and feeding of young, and through indirect routes including shared food and water sources (Forrester et al., 2008; Bunbury et al., 2007), however further studies are required to assess the relative importance of these transmission routes for finches. Establishing the nature and frequency of disease transmission at garden feeders is thus clearly important to identify if mitigation measures are required and, if so, how they should be employed. The greenfinch and chaffinch are both common garden bird visitors in England and Wales across the year, ranking number 9 and 10 in the most frequent garden visitors in the GBW scheme (Cannon et al., 2005). Both species are gregarious, visiting gardens in flocks, but other granivorous passerine species are reported in a comparable number of gardens around feeding stations, for example house sparrows, great tits and blue tits, and they also feed in groups.

More generally, *T. gallinae* is a pathogen of potential significance to the racing pigeon, aviculture, game bird and poultry industries and the implications for finch trichomonosis to these industries remains poorly understood. Continued monitoring of diseases in wild bird populations is required to better quantify and understand their impact on population dynamics (Hudson et al., 1989; Hochachka et al., 2000) and to identify future changes in host-parasite relationships.

CHAPTER 7: PATTERNS OF EPIDEMIC MORTALITY DUE TO TRICHOMONOSIS IN BRITISH PASSERIFORMES, 2005 TO 2007

7.1 INTRODUCTION

Trichomonosis was first recognised as an emerging infectious disease of British finches in 2005, primarily affecting greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*) (Holmes et al., 2005; Pennycott et al., 2005a). Seasonal epidemic mortality occurred in the late summer and early autumn of 2006 over a wide geographical area (Lawson et al., 2006b; Chapter 6).

In this chapter, the species composition of the birds affected by trichomonosis, and the spatial distribution of mortality incidents, in Great Britain during the epidemics of 2006 and the following year (2007) are investigated. Findings are compared between years to identify emergent trends. Evidence for a change in the seasonality of the trichomonosis epidemic between 2006 and 2007 is examined by comparing the month of onset of suspected trichomonosis incidents. The scale of finch mortality that occurred during the epidemic of each year is compared at a local level by examining the number of birds affected per suspected trichomonosis incident.

7.2 MATERIALS AND METHODS

Two independent and complementary reporting schemes were used for surveillance of garden bird mortality across Great Britain: opportunistic reports of garden bird morbidity and mortality solicited from the general public and systematic surveillance via the British Trust for Ornithology's Garden BirdWatch (GBW) participant network (Chapter 3).

Pathological investigations

A standardised post mortem examination (PME) and microbiology protocol was performed on all bird carcasses submitted from both the opportunistic and systematic

surveillance schemes (See Chapter 4). Due to limited resources, some case selection was practised favouring non-finch species during periods of epidemic mortality in an effort to confirm the range of species affected by trichomonosis.

The case definition for trichomonosis comprised necrotic ingluvitis lesions in combination with culture of motile trichomonads and/ or nested PCR amplification (Chapter 6). Cases with characteristic gross lesions from which a *Salmonella* sp. could not be isolated, but in which confirmation of the trichomonosis through culture or nested PCR was not possible, were considered 'suspected trichomonosis'. The total number of confirmed and suspected trichomonosis cases examined post mortem between 1st January 2006 and 30th December 2007 was summarized by species to determine the range of taxa affected by the disease. The geographical distribution of trichomonosis cases examined at post mortem in the greenfinch and chaffinch was compared with that for Columbidae species to assess the degree of overlap and evidence for an epidemiological link in infection between these groups. The distribution of trichomonosis cases in the other affected species (<5 cases examined post mortem) and biodiversity action plan (BAP)-listed species was explored to determine whether spill-over of infection to these species was spatially clustered or widespread.

Trichomonosis incident criteria

Reports of garden bird mortality and morbidity received in 2006 and 2007 were reviewed and criteria employed to designate suspected trichomonosis incidents (Chapter 6). Briefly, a suspected trichomonosis incident was defined, within the 6-month period 1st April to 30th September (inclusive) for each year of study, as mortality including 2 or more dead finches (greenfinch or chaffinch), 1 or more sick finch(es) with typical signs of disease, or if trichomonosis was confirmed post mortem.

Suspected trichomonosis incidents from the opportunistic and systematic reporting schemes were examined to evaluate the species affected by the disease, the temporal and spatial patterns of epidemic mortality that occurred in 2006 and 2007, and the scale of the outbreaks between years. The number of suspected trichomonosis incidents recorded

from the opportunistic surveillance scheme was large; however, this dataset was vulnerable to bias, for example following regional media, particularly affecting geographical or temporal trends. Whilst the systematic surveillance scheme recorded a smaller number of suspected trichomonosis incidents overall, this dataset should be independent from such bias and was evaluated independently to corroborate the findings of the opportunistic scheme. In 2006, reports of dead birds only were available from the systematic GBW network whereas, in 2007, participants provided reports of sick and dead birds in their gardens.

Species affected

The total number of suspected trichomonosis incidents that were classified on the basis of affected greenfinch(es) alone, chaffinch(es) alone, or greenfinches and chaffinches in combination, were expressed as a percentage of the reports within the specified period. Results from the opportunistic and systematic schemes were first compared for each year and, if no significant difference was found, data from both schemes were pooled for analysis and the results between 2006 and 2007 were then compared.

The number of suspected trichomonosis incidents that included columbiform morbidity or mortality (principally wood pigeon (*Columba palumbus*) and collared dove (*Streptopelia decaocto*)), involving 1 or more birds, was determined and the proportions compared in 2006 and 2007.

Since case selection for PME was practised in favour of novel non-finch host species, the species complement of the birds that underwent pathological investigation in which trichomonosis was confirmed could not be used to evaluate the species composition of birds affected during the epidemics. Instead, to evaluate the species affected, the total number of suspected trichomonosis incidents that also included morbidity or mortality of species in which trichomonosis was recorded at PME, other than greenfinches or chaffinches, where the report did not sensibly preclude trichomonosis as the aetiology, was determined and compared between years.

To evaluate whether the number of species affected during a suspected trichomonosis incident increased with the scale of finch mortality, the number of incidents with morbidity or mortality involving species in which trichomonosis was recorded at PME, other than greenfinches or chaffinches where the report did not sensibly preclude trichomonosis as the aetiology, was determined and compared with the total number of finches (greenfinch and chaffinch combined) found dead using Pearson's correlation.

Temporal trends

For the opportunistic data, the month of onset when finch morbidity or mortality was first observed was summarised for the suspected trichomonosis incidents in both years. The systematic scheme data was independently examined in the same manner to corroborate the findings and identify temporal bias within the opportunistic dataset.

Spatial trends

The distribution of suspected trichomonosis incidents was determined for 2006 and 2007 for the opportunistic dataset overall, on the basis of the scale of finch mortality and progression with time through the specified season. The systematic scheme data was independently examined in the same manner to corroborate the findings and identify spatial bias within the opportunistic dataset.

Scale of mortality

The scale of mortality was assessed at the local level by examining the number of dead finches reported per suspected trichomonosis incident, and comparing this between 2006 and 2007. The total number of dead greenfinches and/ or chaffinches per suspected trichomonosis incident was classified according to the following classes; 0 birds, 1 bird, 2 to 5 birds, 6 to 10 birds, 11 to 20 birds and over 21 birds. Results from the opportunistic and systematic schemes were compared between years and, if no significant difference was found, data from both schemes were pooled for these analyses.

Non-parametric analyses including the Pearson chi-square test and binomial test of proportions were performed as appropriate, unless otherwise indicated, using SPSS 17.0 for Windows (SPSS inc., Chicago, USA) and R–CRAN (http://www.R-project.org). Spatial data were presented using ArcView 3.0 geographical information system (GIS) software (ESRI GIS and Mapping Software, California, U.S.A.).

7.3 RESULTS

Pathological investigations

In total, 1,238 birds comprising 48 species and 24 families were examined post mortem between 1st January 2006 and 31st December 2007. Trichomonosis was confirmed as, or suspected to be, the cause of death in 518 birds of 17 species from 313 sites (Table 7.1). Species that had 10 or more individuals examined post mortem, from which no cases of trichomonosis were suspected or confirmed, are also detailed in Table 7.1. Of the 439 confirmed cases of trichomonosis, diagnosis was made on the basis of necrotic ingluvitis lesions in combination with culture of trichomonads only in 73 birds; on the basis of necrotic ingluvitis lesions with nested PCR amplification only in 270 birds and on the basis of culture and nested PCR amplification in 96 birds. Fringillidae and Columbidae species accounted for 94.4% of all the trichomonosis cases, although the disease was confirmed or suspected in other passerine species, belonging to the Paridae, Passeridae, Prunellidae and Turdidae families.

Trichomonosis cases in Columbidae species were widely distributed in both 2006 and 2007 (Figure 7.1b & d). The regions with the greatest number of trichomonosis cases in greenfinches and chaffinches (Figure 7.1a & c) did not coincide with a cluster of columbiform cases. Indeed, c. 45% (9/20) of the sites from which columbiform trichomonosis cases were submitted for PME in 2006 were from the south-east and eastern counties of England, regions with minimal contemporaneous finch mortality in that year. The distribution of trichomonosis cases in BAP-listed species (bullfinch and house sparrow) and other species (dunnock and goldfinch) showed no evidence of spatial clustering (Figure 7.2).

Table 7.1: Trichomonosis cases confirmed/suspected at PME, 1st January 2006 to 30th December 2007.

Order / Family / Species	Trichomonosis cases (Confirmed/ Suspected)	Total number of PME
Accipitrformes		
Accipitridae		
Sparrowhawk *Accipter nisus*	2 (2/ 0)	6
Common buzzard *Buteo buteo*	2 (2/ 0)	2
Columbiformes		
Columbidae		
Collared dove *Streptopelia decaocto*	21 (19/ 2)	27
Wood pigeon *Columba palumbus*	23 (21/ 2)	32
Passeriformes		
Fringillidae		
Brambling *Fringilla montifringilla*	1 (1/ 0)	2
Bullfinch *Pyrrhula pyrrhula*	8 (7/ 1)	27
Chaffinch *Fringilla coelebs*	140 (101/ 39)	223
Goldfinch *Carduelis carduelis*	10 (9/ 1)	49
Greenfinch *Carduelis chloris*	278 (245/ 33)	422
Siskin *Carduelis spinus*	3 (3/ 0)	70
Yellowhammer *Emberiza citrinella*	5 (5/ 0)	10
Paridae		
Blue tit *Cyanistes caeruelus*	1 (0/ 1)	21
Great tit *Parus major*	3 (3/ 0)	27
Passeridae		
House sparrow *Passer domesticus*	10 (8/ 2)	75
Prunellidae		
Dunnock *Prunella modularis*	9 (9/ 0)	36
Sturnidae		
Starling *Sturnus vulgaris*	0 (0/0)	31
Turdidae		
Blackbird *Turdus merula*	1 (1/ 0)	73
Robin *Erithacus rubecula*	0 (0/0)	25
Song thrush *Turdus philomelos*	0 (0/0)	18
Piciformes		
Picidae		
Great spotted woodpecker *Dendrocopus major*	0 (0/0)	10
Strigiformes		
Strigidae		
Tawny owl *Strix aluco*	1 (1/ 0)	2

Figure 7.1a-d: Distribution of trichomonosis cases based on PME data from 1st January 2006 to 31st December 2007.

(a) Greenfinch and chaffinch 2006

(b) Columbiform species 2006

(c) Greenfinch and chaffinch 2007

(d) Columbiform species 2007

Figure 7.2 a-d: Distribution of trichomonosis cases based on PME data from 1st January 2006 to 31st December 2007. (a) Bullfinch (b) Dunnock (c) Goldfinch (d) House sparrow.

Bullfinch

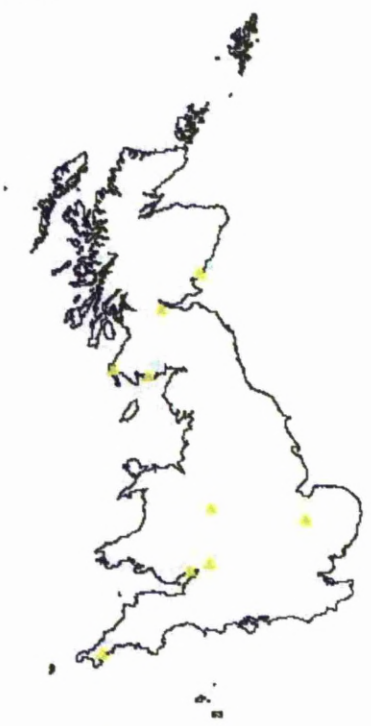
(a)

Dunnock

(b)

Goldfinch

(c)



House sparrow

(d)

Trichomonosis incident data

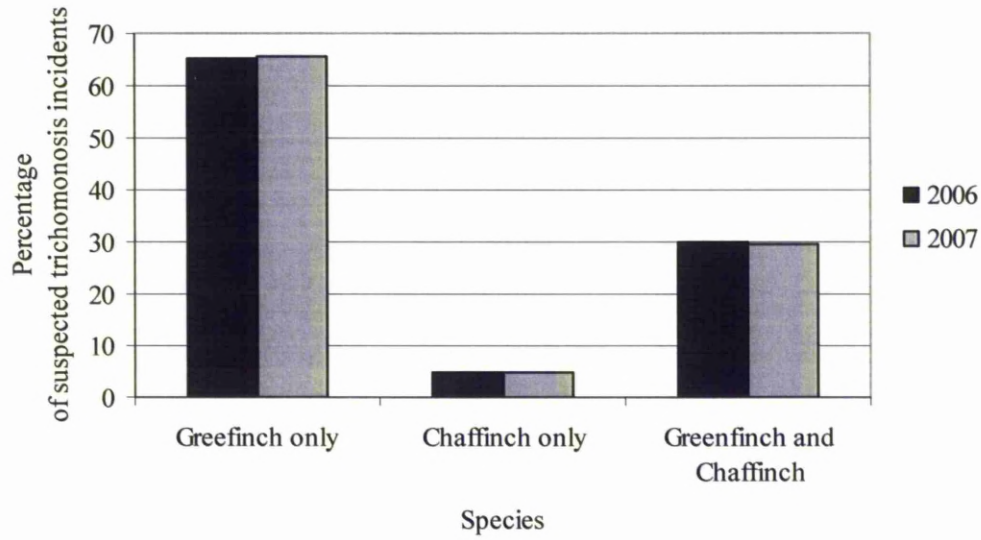
A total of 1,054 suspected trichomonosis incidents, according to the incident definition, were recorded between 1st April and 30th September 2006, corresponding to 55.1% (n=1912) of all garden bird mortality and morbidity reports collated through the opportunistic reporting scheme during 2006. For the same period in 2007, a total of 1,505 suspected trichomonosis incidents were recorded, accounting for 66.2% (n=2275) of all reports. The proportion of opportunistic reports identified as suspected trichomonosis incidents was significantly greater in 2007 than 2006 (X^2 =52.707, df=1, P<0.0001).

For the systematic surveillance scheme, suspected trichomonosis incidents during the specified interval were determined on the basis of dead bird reports available for both 2006 and 2007, enabling direct comparison between years with consistent methodology. In 2006, 7.3% (39 of 674) of sites monitored during the specified interval reported trichomonosis incidents as compared with 7.4% (47 of 632) of sites in 2007; there was no significant difference in the proportion of monitored sites reporting trichomonosis incidents between years (X^2=1.188, df=1, P>0.05). For the systematic surveillance scheme in 2007, when the reports of both dead and diseased birds were considered, 15.7% (99 of 632) of sites had suspected trichomonosis incidents.

Species affected

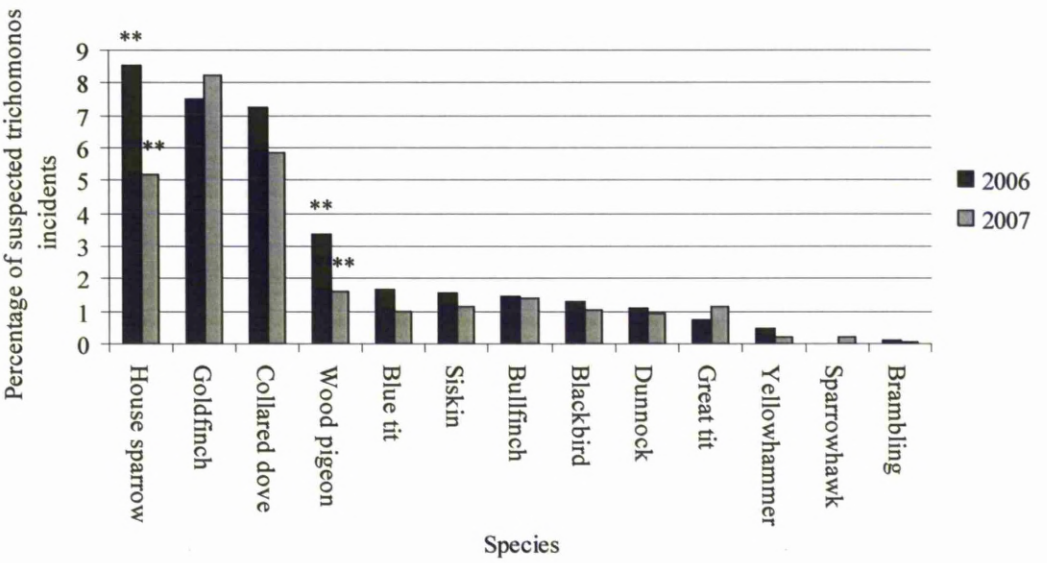
There was no significant difference in the finch species (greenfinch and/ or chaffinch) involved in the suspected trichomonosis incidents between the opportunistic and systematic datasets, for 2006 (X^2=4.434, df=2, P=0.109) and 2007 (X^2=3.887, df=2, P=0.143) respectively; consequently the data were pooled for the analysis comparing the results from both years. There was no significant difference (X^2=4.285, df=2, P=0.117) between the number of suspected trichomonosis incidents that were identified on the basis of greenfinch mortality alone, chaffinch mortality alone, or where both species were involved, between 2006 and 2007: the results for both years were very similar and greenfinches were most commonly involved (Figure 7.3).

Figure 7.3: Percentage of suspected trichomonosis incidents with morbidity and/ or mortality of greenfinches or chaffinches alone, or both species in combination, for 2006 and 2007.



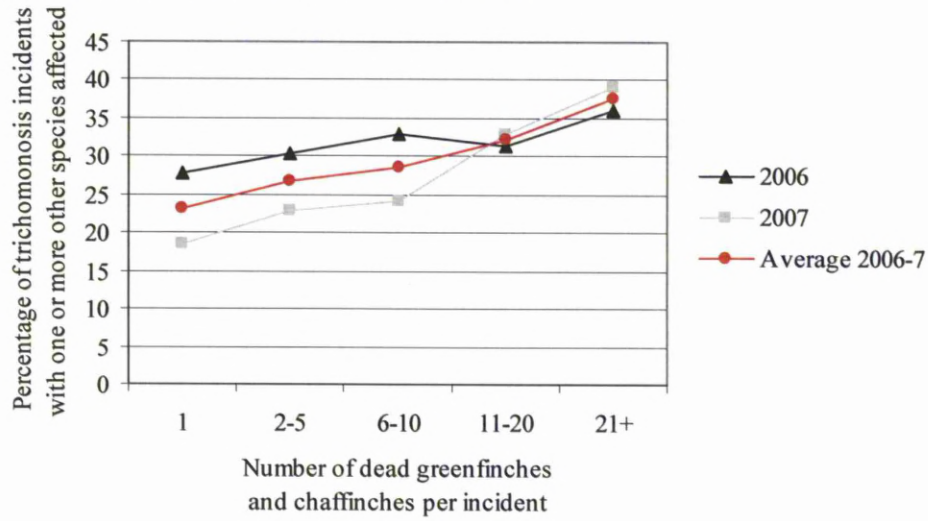
The percentage of suspected trichomonosis incidents that involved contemporaneous morbidity or mortality of species (that have been confirmed to be affected by trichomonosis through PME), other than the greenfinch and chaffinch, is shown in Figure 7.4, considering the pooled opportunistic and systematic datasets. There were significantly fewer incidents involving the house sparrow (X^2=11.3061, df=1, P=0.0008) and the wood pigeon (X^2= 8.112, df=1, P=0.004) in 2007 than 2006; however, no significant change between years was seen in the other species. Concurrent morbidity or mortality of columbiform species occurred in a significantly greater percentage of incidents in 2006 (9.9% - 108 of 1093) than in 2007 (7.3% - 117 of 1604) considering the pooled opportunistic and systematic datasets (X^2=5.3553, df =1, P=0.02).

Figure 7.4: Percentage of suspected trichomonosis incidents with concurrent morbidity and/ or mortality of other species known to be affected by trichomonosis, for 2006 and 2007.
** denotes a significant difference between years at P<0.01.



The number of suspected trichomonosis incidents that involved contemporaneous morbidity or mortality of species (that have been confirmed with trichomonosis on PME), other than the greenfinch and chaffinch, is shown as a percentage of the number of incidents for each of the mortality classes (total number of dead greenfinches and chaffinches (combined)) (Figure 7.5). Incidents from the opportunistic and systematic surveillance schemes were pooled for these analyses. A significant positive association was found (Pearson's correlation coefficient r=0.977, P=0.04) with species other than the greenfinch and chaffinch being more likely to be affected at sites with high finch mortality.

Figure 7.5: Percentage of suspected trichomonosis incidents with morbidity or mortality of 1 or more species other than greenfinch or chaffinch.

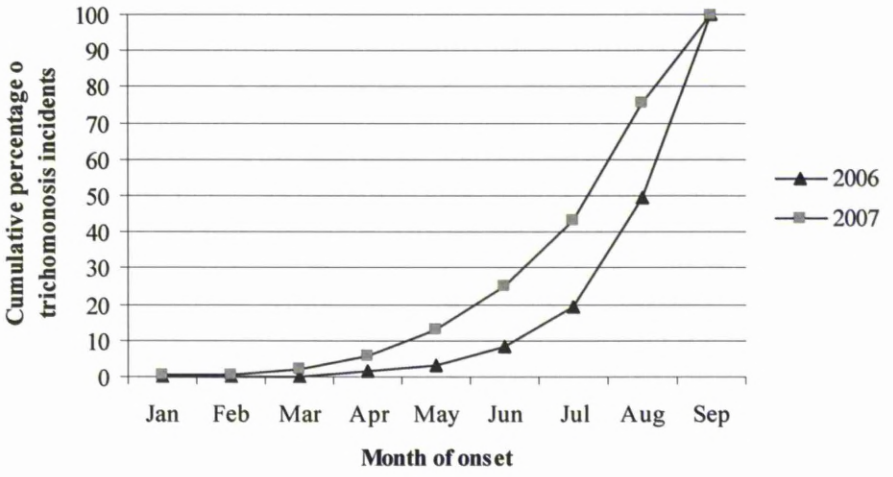


Temporal trends

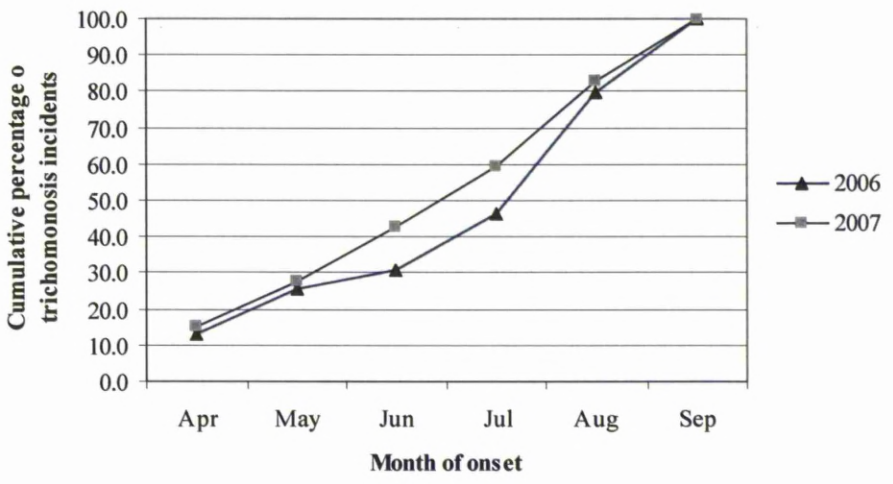
The temporal distribution of the month when finch morbidity or mortality was first observed from the opportunistic dataset is shown in Figure 7.6. Month of onset was considered from January to September inclusive for suspected trichomonosis incidents with morbidity or mortality that occurred between 1st April and 30th September inclusive. Incidents with onset between October and December were excluded from this summary to exclude the winter months when salmonellosis incidents, the most common differential diagnosis for multiple finch mortality events in Great Britain based on PME, are known to occur. There was a significant difference between 2006 and 2007 for the opportunistic dataset (X^2=215.603, df=5, P=0.000) with a greater percentage of trichomonosis incidents reporting onset earlier in the year in 2007. Review of the systematic dataset (considering month of onset from April and September) found no evidence for a significant difference in the temporal distribution of trichomonosis incidents between years (X^2=2.936, df=5, P=0.710), however, the direction of trend supported that seen in the opportunistic data.

Figure 7.6: Temporal distribution of trichomonosis epidemic in 2006 and 2007 using (a) opportunistic and (b) systematic datasets.

(a)



(b)



Spatial trends

In 2006, the trichomonosis incidents from the opportunistic dataset show the greatest concentration in the central, western and south-western counties of England: a similar trend was observed in the systematic data (Figure 7.7a-b).

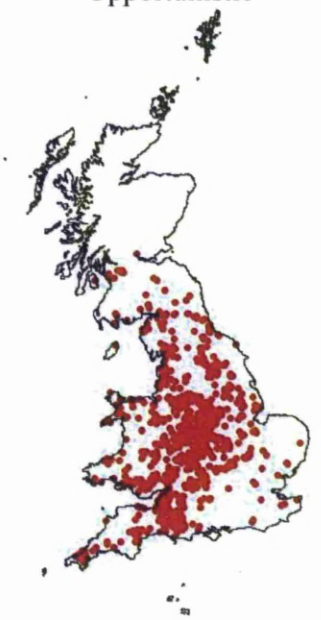
In 2007, the opportunistic dataset indicated a marked shift in distribution with the greatest density of suspected trichomonosis incidents in the southern and eastern counties of England; the south-west continued with high numbers of incidents, however, the reports from the Midlands region markedly declined (Figure 7.7c). The distribution of suspected trichomonosis incidents from the systematic dataset assigned on reports from dead birds only, or from sick and dead birds, revealed similar widespread distribution across England and Wales: consequently all maps presented subsequently in this chapter consider the entire systematic dataset. The systematic data for 2007 mirror the eastward spread seen in the opportunistic dataset, with incidents being reported from East Anglia and the southern counties of England (Figure 7.7d). However, the systematic incidents do not show the same high concentration of reports as the opportunistic dataset, rather they are diffusely distributed across the regions affected by trichomonosis in 2006 and 2007. Both datasets indicate further spread into Scotland in 2007.

Figure 7.7: Distribution of suspected trichomonosis incidents from the opportunistic and systematic schemes, 2006 and 2007.
Red dots represent trichomonosis incidents. Black dots in Figure 7.7b & d represent monitored GBW sites.

Opportunistic

2006

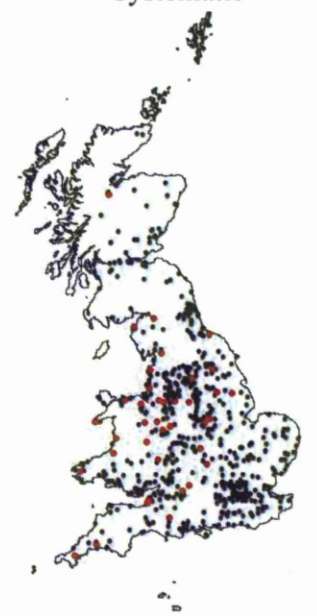
(a)



Systematic

2006

(b)



2007

(c)

2007

(d)

The distribution of suspected trichomonosis incidents in the opportunistic dataset for each of the finch mortality classes (Figures 7.8-7.11) showed a similar spread across the regions most affected, for both 2006 and 2007, with no exaggerated clustering of the sites with greatest recorded mortality. The distribution of suspected trichomonosis incidents in the systematic dataset for each of the finch mortality classes in 2006 was consistent with those of the opportunistic dataset, peaking in the central, western and south-western counties of England. In 2007, however, trichomonosis incidents in the systematic dataset for each of the finch mortality classes were evenly spread across England and Wales, with no exaggerated clustering of the sites with greatest recorded mortality.

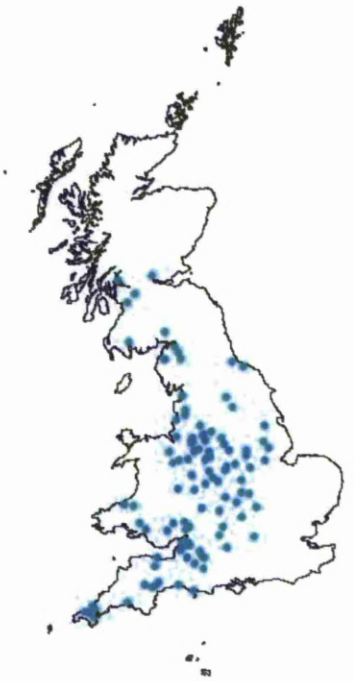
Figure 7.8: Distribution of suspected trichomonosis incidents with **1 dead finch** per site from the opportunistic and systematic schemes, 2006 and 2007.

Opportunistic

Systematic

2006

(a)

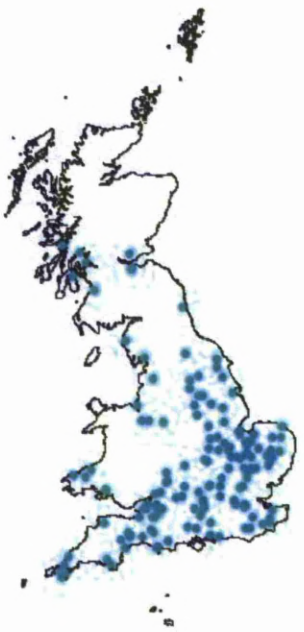


2006

(b)

2007

(c)



2007

(d)

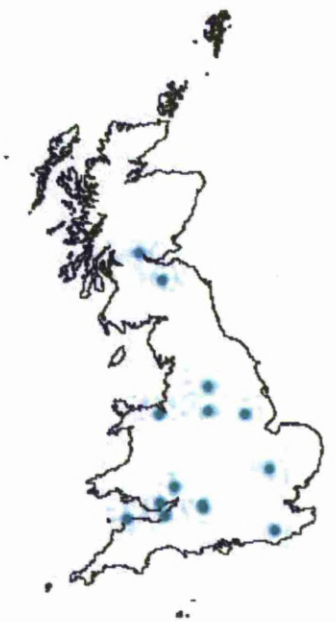
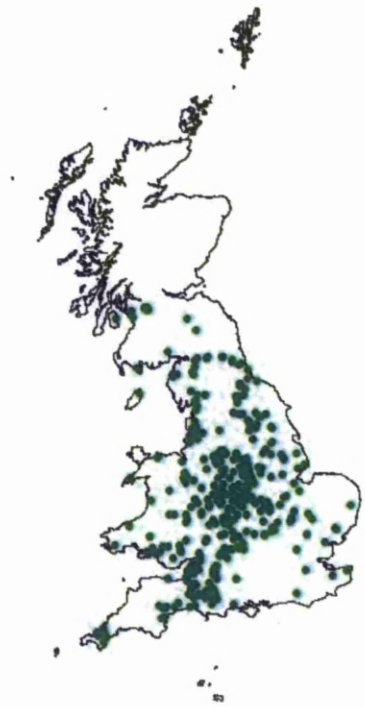


Figure 7.9: Distribution of suspected trichomonosis incidents with **2 to 5 dead finches** per site from the opportunistic and systematic schemes, 2006 and 2007.

Opportunistic

2006

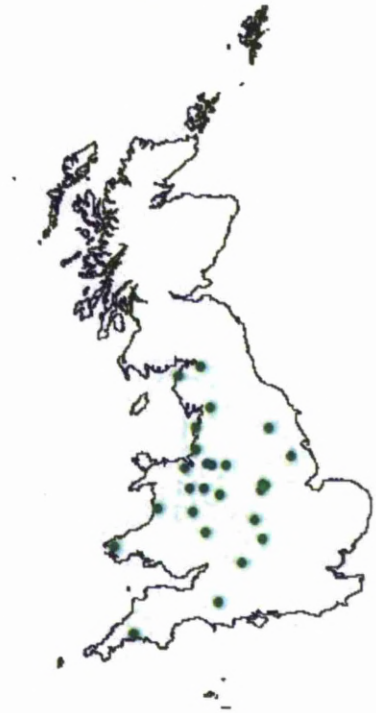
(a)



Systematic

2006

(b)



2007

(c)

2007

(d)

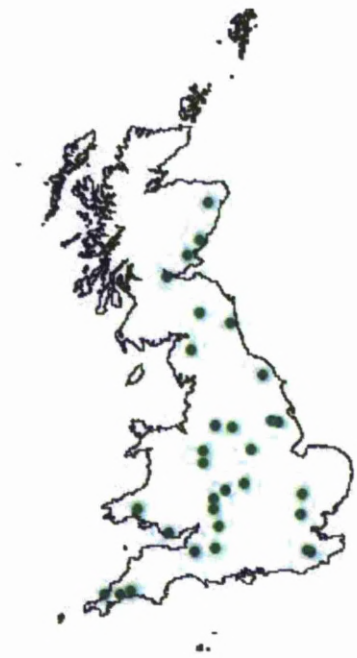
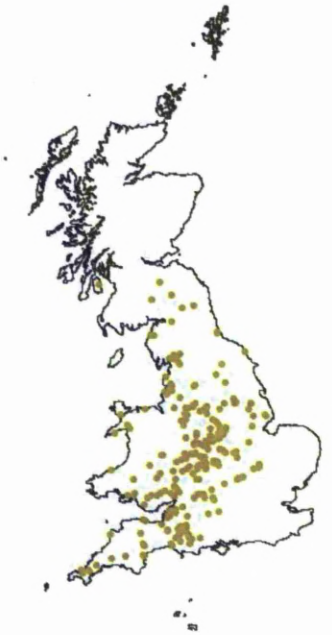


Figure 7.10: Distribution of suspected trichomonosis incidents with **6 to 10 dead finches** per site from the opportunistic and **6 or more dead finches** per sites from the systematic scheme.

Opportunistic

2006

(a)



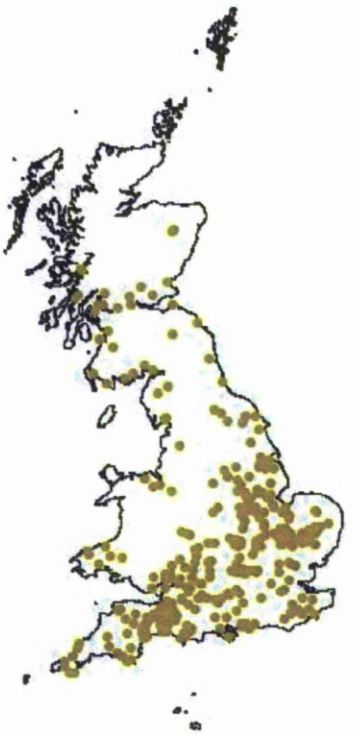
Systematic

2006

(b)

2007

(c)



2007

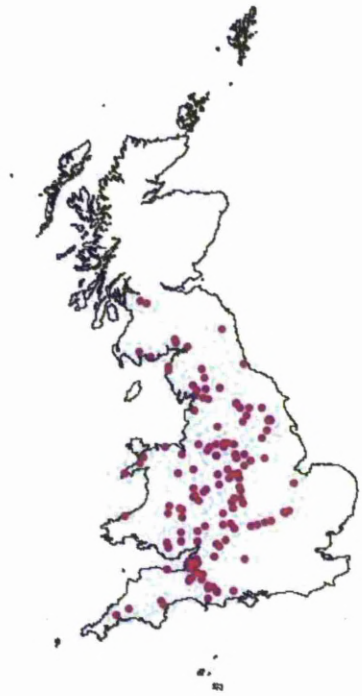
(d)

Figure 7.11: Distribution of suspected trichomonosis incidents with **11 to 20, or 21 or more, dead finches** per site from the opportunistic scheme, 2006 and 2007.

11 to 20 dead finches

2006

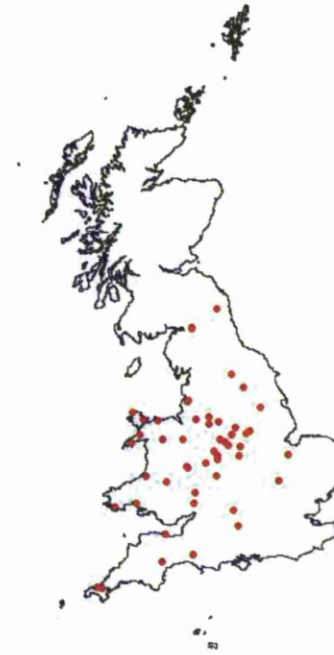
(a)



21 or more dead finches

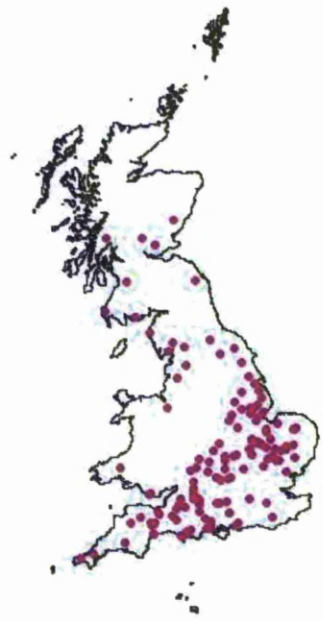
2006

(b)



2007

(c)



2007

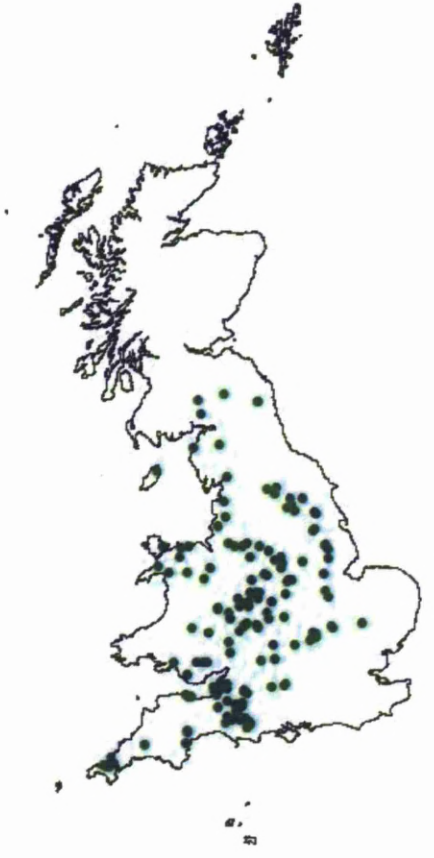
(d)

In 2006, the opportunistic dataset of suspected trichomonosis incidents showed some evidence of spread into East Anglia by the months of onset August-September, consistent with eastward spread with time (Figure 7.12); no similar trend was observed in the systematic dataset (Figure 7.13). In 2007, the opportunistic dataset showed an increased number of trichomonosis incidents in England in the region south of a line between the Bristol Channel and The Wash with onset August-September (Figure 7.14). For the systematic dataset in 2007, trichomonosis incidents were distributed across Great Britain with no evident temporal trends in the period of onset April – September (Figure 7.15).

Figure 7.12a-c: Distribution of suspected trichomonosis incidents from the opportunistic dataset in 2006 by month of onset.

(a) April and May

(b) June and July



(c) August and September

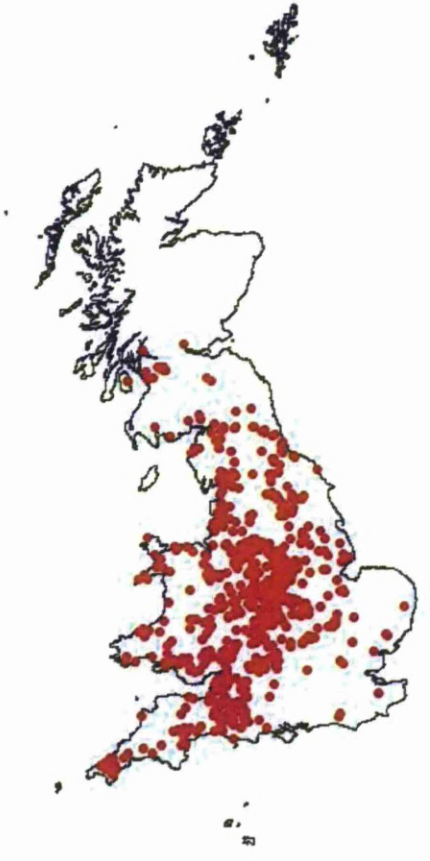
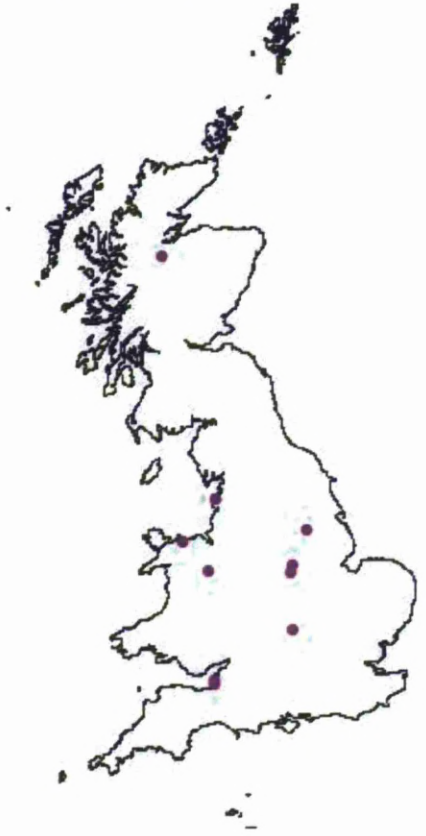
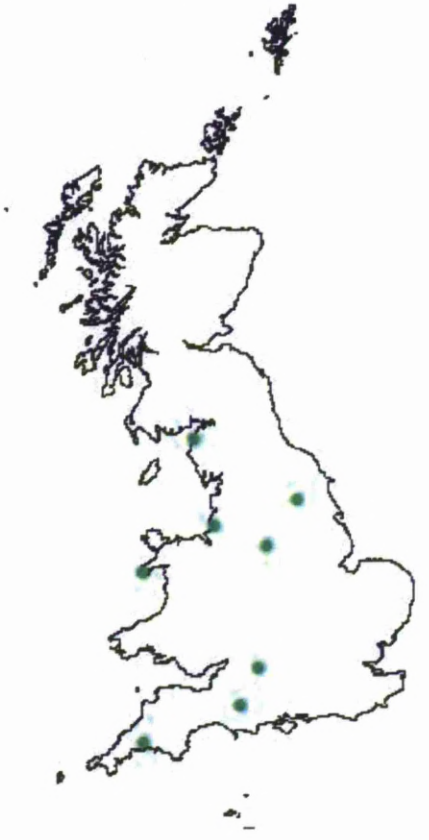


Figure 7.13a-c: Distribution of suspected trichomonosis incidents from the systematic dataset in 2006 by month of onset

(a) April and May



(b) June and July



(c) August and September

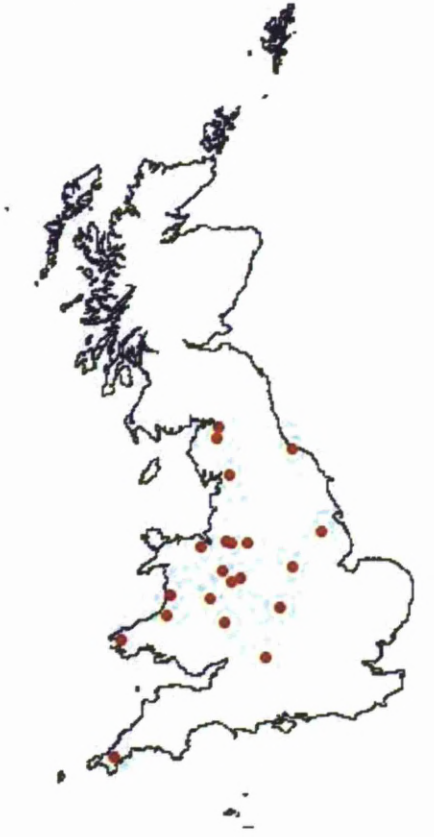
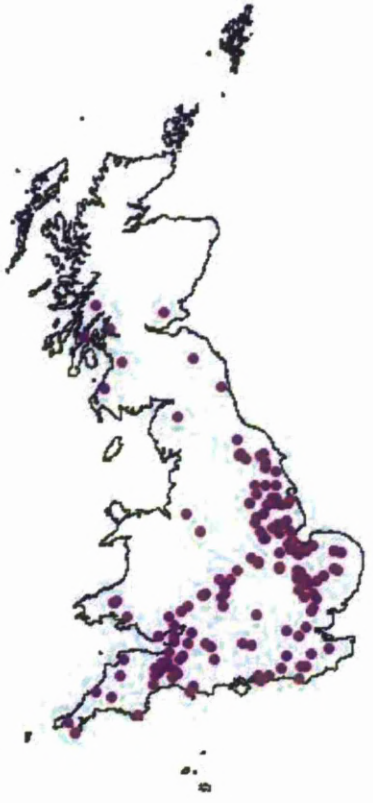


Figure 7.14a-c: Distribution of suspected trichomonosis incidents from the opportunistic dataset in 2007 by month of onset.

(a) April and May

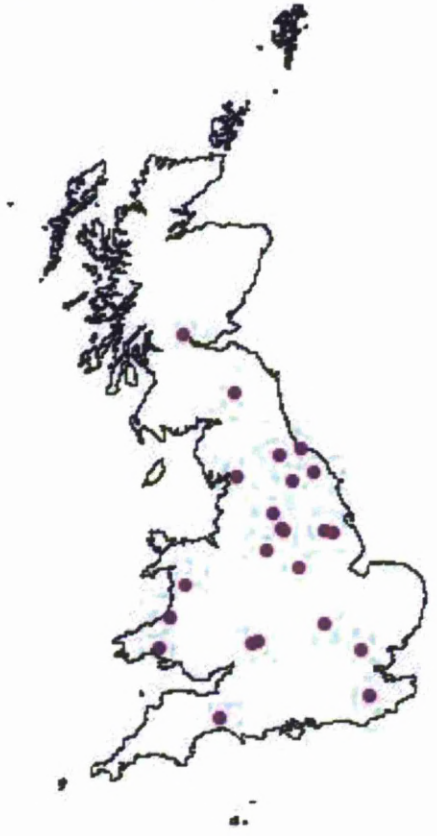


(b) June and July

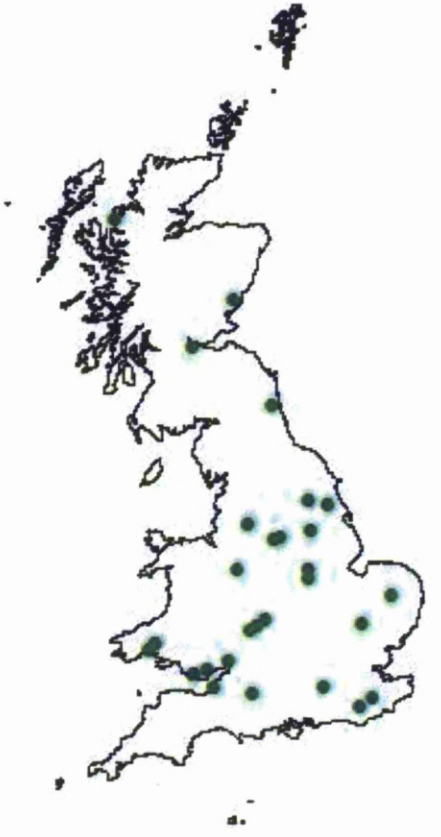
(c) August and September

Figure 7.15a-c: Distribution of suspected trichomonosis incidents from the systematic dataset in 2007 by month of onset.

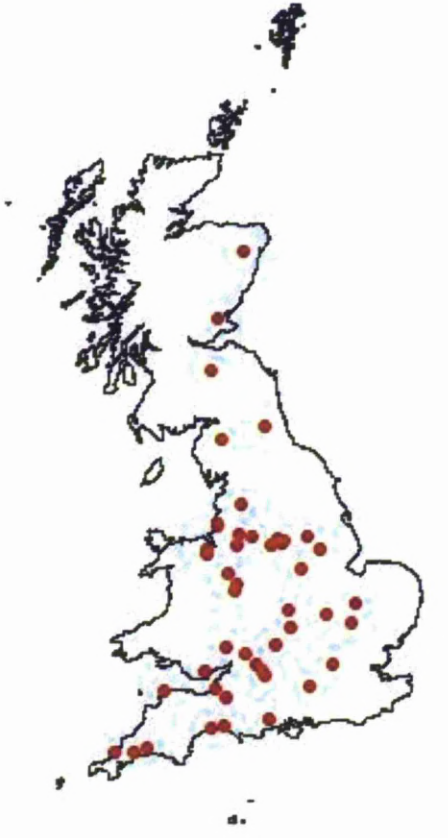
(a) April and May



(b) June and July



(c) August and September

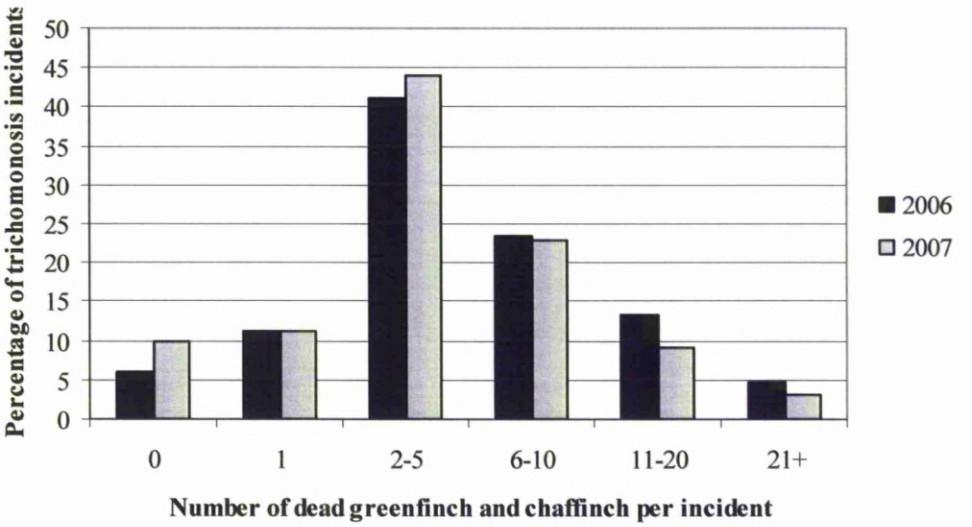


Scale of mortality

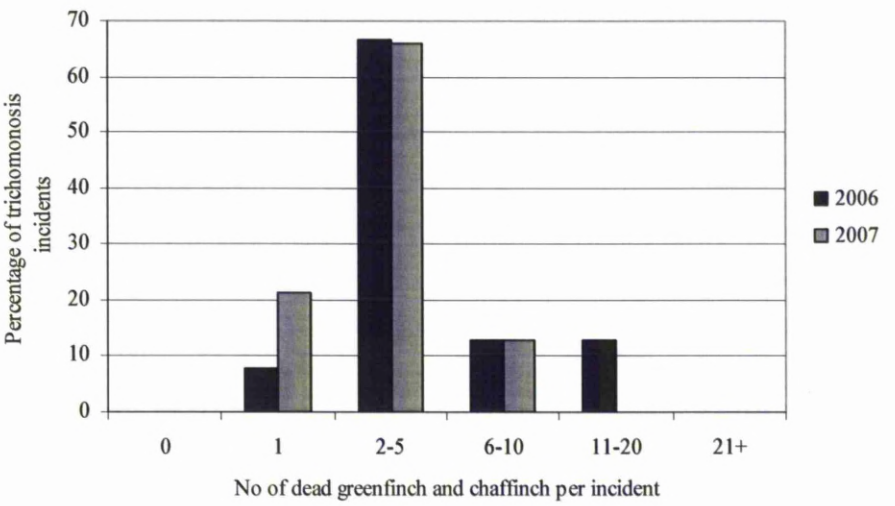
The number of dead finches reported per suspected trichomonosis incident, for the opportunistic and systematic datasets, is expressed as a percentage of the total number of incidents in 2006 and 2007 in Figure 7.16. There was a significant difference in the number of dead finches per trichomonosis incident reported in the opportunistic and systematic datasets, for both 2006 (X^2=14.671, df=2, P=0.002) and 2007 (X^2=14.641, df=2, P=0.001), with the greater percentage of sites experiencing high mortality reported in the opportunistic dataset. Comparison between years for the opportunistic dataset identified significantly greater site mortality in 2006 than 2007 (X^2=26.178, df=5, P=0.000); however, no significant difference was found between 2006 and 2007 for the systematic datasets (X^2=4.503, df=2, P=0.105).

Figure 7.16: Number of dead finches per incident for suspected trichomonosis cases in 2006 and 2007 for (a) opportunistic and (b) systematic data.

(a)



(b)



7.4 DISCUSSION

Species affected

Trichomonosis was confirmed in a total of 16 species in Great Britain and accounted for over 40% of all PME submissions during 2006 and 2007. Suspected trichomonosis incidents, based on the incident definition, involved greenfinches in the majority of cases, in combination with chaffinches in approximately one third of the cases, but rarely involved chaffinches alone. This is reflected by the greater decline in the 2007 breeding populations of greenfinches, than chaffinches, in areas of high trichomonosis incidence in 2006 (Chapter 6). No significant difference in the percentage of greenfinch or chaffinches involved in the epidemics was seen between years.

Sympatric columbiform species are postulated to be the most likely origin of the *T. gallinae* strain affecting British finches. In California, Anderson et al. (2009) hypothesise that endemic infection in the house finch population results from repeated spill-over events from sympatric columbiforms, and that close proximity at shared food and water sources may facilitate this transfer. In the current study, concurrent columbiform morbidity or mortality was seen in a minority of trichomonosis incidents with a significant reduction between 2006 and 2007. The PME data show that the columbiform cases of trichomonosis do not coincide spatially with the regions of peak finch mortality due to the disease. Whilst the origin of the parasite in Great Britain may have been wild columbiforms, and interspecific transfer of infection between Fringillidae and Columbidae species is likely, this study supports a contrasting epidemiology to the situation in North America with direct transmission between finches propagating the epidemics in Great Britain. Disease transmission within finch populations will be facilitated by their highly gregarious nature and flock-feeding behaviours (Newton 1972).

This is the first report of trichomonosis infecting wild birds within the Prunellidae, Paridae and Turdidae. There was no evidence to support a change in the species composition between 2006 and 2007 for the majority of species and the trichomonosis

incidents which reported their involvement were geographically widespread, rather than clustered: the most consistent explanation for these observations is that multiple, isolated spill-over events from finches led to infection in these species. Parasite spread from finches to other garden bird species seems most likely to occur through shared food and water sources, for example feeding stations that are frequently used in British gardens. The majority of passerine species in which trichomonosis was confirmed feed on seed or nuts from hanging feeders or bird tables (i.e., blue tit, brambling, bullfinch, great tit, goldfinch, house sparrow, siskin, yellowhammer), typically in groups.

Trichomonosis was diagnosed in a small number of dunnocks which, although principally insectivorous, will take seed (Toms 2003). Unusually trichomonosis was also confirmed in a single blackbird, a species considered to be insectivorous and frugivorous. Spill-over of the parasite through shared water, rather than food, sources offers the most likely explanation for the infection in this case. Bunbury et al. (2007) successfully cultured *T. gallinae* from water sources used by Mauritian columbids demonstrating the potential for parasite transmission via this route.

The trichomonosis epidemic of 2006 caused a marked reduction in the population of Great Britain's greenfinches and chaffinches, particularly in the areas of peak mortality (Chapter 6). Trichomonosis was confirmed in a number of species of conservation concern with dedicated BAPs (e.g. bullfinch, house sparrow and yellowhammer), however, only a small proportion of finch trichomonosis incidents involved concurrent morbidity or mortality in these species. Of these, the house sparrow was most frequently involved in both years although the number of suspected trichomonosis incidents including this species was significantly reduced in 2007.

This study indicates that the likelihood of morbidity or mortality affecting species other than greenfinches or chaffinches in a trichomonosis incident is increased by the local scale of the outbreak (based on the total number of dead greenfinches and chaffinches). This supports the hypothesis that disease in species other than the greenfinch and chaffinch is more likely to occur at sites with large numbers of affected finches,

implying that this occurs as a result of local transmission through contact at shared food or water sources, rather than independently circulating in those wild bird populations at a low level. The unexpectedly wide range of species susceptible to the parasite warrants further monitoring of the birds affected since it is not currently possible to predict the likelihood that the parasite may, once again, establish in a novel avian family.

Temporal trends

The trichomonosis epidemic of both years had a seasonal distribution with onset of morbidity or mortality reported to occur in the majority of incidents between April and September inclusive. The opportunistic dataset indicates that the epidemic began earlier in 2007, with the peak number of incidents reported to have onset in August, rather than September as in 2006. Whilst no significant difference between the temporal distribution of trichomonosis incidents was found through the systematic scheme, the data support the same trend indicating that this observation may be genuine rather than the result of bias within the opportunistic dataset. Various factors may have influenced the timing of the epidemic, for example climatic variables, food availability and the timing of the breeding season. The weather in the British summer of 2006 and 2007 contrasted starkly, with a dry and hot summer in 2006 followed by a wet and cold summer in the following year (Met Office 2009); however, since the available climatic data is summarized by month and region it is not possible to investigate daily variation in local climatic conditions at the sites where disease incidents occurred. Epidemic mortality occurred in both years suggesting that any impact of climate only influenced timing to a certain extent and that the size of the susceptible population of finches, including näive juveniles in the breeding season, and behavioural changes that occur in early summer facilitating transfer of the parasite between individuals, for example adults feeding young and visiting garden bird feeding stations, may explain the seasonality to a greater extent.

Scale of mortality

This study found that the number of dead finches reported across trichomonosis incidents was greater for the opportunistic than systematic scheme in both years: this

finding is logical since members of the public would have been most likely to report their observations opportunistically if they found several dead birds as compared with the systematic participants who were asked to observe the birds in their garden and report all abnormalities (Chapter 3). The proportion of trichomonosis incidents reporting high levels of finch mortality in the opportunistic dataset reduced significantly in 2007. It is plausible that an increased profile of trichomonosis in the second year of epidemic mortality led to an increased number of people opportunistically reporting observations of sick or single dead finches in their garden. However, no significant difference was seen between years with the systematic dataset.

The modal group was 2-5 dead finches per trichomonosis incident; however, it was not unusual for in excess of 20 dead finches to be reported at an individual site in the opportunistic dataset. Due to the strong likelihood of underreporting of mortality based on recovery of wild animal carcasses (Wobeser et al., 1992), only a small proportion of deaths are likely to have been observed, even at monitored sites; consequently the reports are likely to reflect very high levels of mortality in the local finch population.

Whilst the overall number of trichomonosis incidents recorded through the opportunistic scheme was greater in 2007 than 2006, direct comparison between years was complicated by the potential for bias following regional or national media. Importantly, the systematic data indicated that the percentage of monitored sites that reported trichomonosis incidents was remarkably similar between both years. Further decline of the greenfinch and chaffinch populations is predicted following the second year of epidemic mortality: assessment of the impact of the 2007 trichomonosis epidemic on the breeding population of birds is required using the approach described in Chapter 6.

CHAPTER 8: CLONAL STRAIN OF *TRICHOMONAS GALLINAE* ACCOUNTS FOR EMERGING DISEASE THREAT TO BRITISH BIRDS

8.1 INTRODUCTION

Trichomonosis was first recognised as an emerging infectious disease (EID) of British finches in 2005 (Pennycott et al., 2005; Holmes et al., 2005) and caused epidemic mortality in subsequent years (Chapter 6). The reason why *Trichomonas gallinae*, an endemic parasite of columbid populations, caused novel disease in sympatric finches is not understood. Explanatory hypotheses include altered host factors, for example increased rates of inter-specific contact at shared feeding sources may have led the parasite to spill-over to a näïve host population. Alternatively, genetic mutation of an endemic strain(s) of *T. gallinae* might have occurred, leading to the emergence of an increasingly virulent strain with a wider range of susceptible host species. Genotyping studies of *T. gallinae* parasites derived from a range of avian hosts, before and after the emergence of finch trichomonosis, were performed to explore the latter hypothesis and are the focus of this chapter.

Sequence analysis of the Internal Transcribed Spacer (ITS)1/ 5.8S rRNA/ ITS 2 region of *Trichomonad* sp. parasites isolated from greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*) that had died as a result of trichomonosis in Great Britain (2005 – 2006) confirmed the species identification as *T. gallinae* (Chapter 6) with 100% homology to published columbiform isolates (EU215369, Gerhold et al., 2008; EF208019, Gaspar da Silva et al., 2007; AY349182, Kleina et al., 2004) and passerine isolates from North America (EU290649, Anderson et al., 2009). A nested PCR targeting a specific fragment from the trichomonad small subunit (SSU) rRNA gene was developed for case diagnosis (Chapter 6) and the 149 base pair (bp) sequence obtained matched published isolates from columbiform species with 100% homology (EU215373, EU215374; Gerhold et al., 2008). No sequence variation between isolates collected from greenfinch and chaffinch species in 2005 or 2006 was detected in the

ITS1/ 5.8S rRNA/ ITS 2 region or the SSU rRNA gene although only 9 cases were examined for each bird species (Chapter 6).

In this chapter, published techniques for phylogenetic sequence analysis based on a non-coding (ITS1/ 5.8S rRNA/ ITS 2) region and coding genes (SSU rRNA and Fe-hydrogenase) were used to investigate strain variation between *T. gallinae* isolates collected from multiple British bird species. Studies have used these markers for species identification (Kleina et al., 2004; Gaspar da Silva et al., 2007) but found minimal sequence variation between isolates collected from different birds and regions (Gerhold et al., 2008; Anderson et al., 2009): consequently these techniques may not provide adequate resolution to differentiate between parasite strains.

Since published techniques for sequence analysis have not enabled fine-scale strain variation between *T. gallinae* isolates to date, random amplified polymorphic DNA (RAPD) analyses have been used for this purpose (Gaspar da Silva et al., 2007). RAPD analyses, however, demand stringent and consistent methodologies and DNA quality to ensure that repeatable results are obtained both between gels and between samples. Whilst microsatellite studies would be desirable in the future, none are currently characterised for use within the Trichomonadidae.

In this study, available methodologies for detection of *T. gallinae* parasite strain variation were optimised and novel approaches developed. First, the protocol for RAPD analysis published by Gaspar da Silva et al. (2007) was modified by using FAM-labelled oligonucleotide probe database (OPD) primers and automated reading to reduce gel-gel variation and improve accuracy. A novel clustering analysis tool was constructed to enable meta-analysis of the RAPD results from multiple OPD primer sets in combination to improve the power of the analysis.

Second, sequence analysis of a new typing locus, the coding Fe-hydrogenase gene, was used. This house-keeping gene has been used successfully to evaluate relationships between various organisms, including *T. vaginalis* (Voncken et al., 2002) and shows

potential as a novel genotyping marker with the capacity to detect fine-scale variation between trichomonad parasites.

Results from the sequence and RAPD analyses were appraised in combination. Evidence for temporal and geographical genotypic variation of isolates was evaluated, along with variation that may be associated with strain virulence. Isolates from columbiform species collected before and after emergence of trichomonosis in British finches were examined to evaluate whether spill-over from pigeons or doves provides the most plausible explanation for the origin of the parasite strain affecting finches.

8.2 MATERIALS AND METHODS

DNA extraction techniques for PCR and sequencing

DNA was extracted from frozen/thawed necrotic ingluvitis lesions (20-25 mg) collected from trichomonosis cases using the Biosprint 15 DNA Blood Kit (Qiagen, UK) for purification of DNA from tissue according to the manufacturer's instructions. Alternatively, DNA was extracted from culture media with viable organisms, or from positive culture media archived at -20 °C. Parasites were pelleted by centrifugation for 3 minutes at 14,500 rpm before DNA extraction using the same technique. A reference strain *Trichomonas gallinae* (Rivolta) Stabler (American Type Culture Collection (ATCC) Number 30230) was purchased from the ATCC and DNA was extracted from live culture. A trichomonad parasite was cultured from the caecal contents of a pheasant (*Phasianus colchichus*) (B306356) using an In-Pouch kit (BioMed Diagnostics, San Jose, U.S.A.) and DNA was extracted from live culture.

PCR for the ITS1/ 5.8S rRNA / ITS2 region

PCR was used to amplify the non-coding ITS1/5.8S rRNA/ITS2 region using the TFR1 and TFR2 primers described by Gaspar da Silva et al. (2007) with an adapted protocol (Chapter 6). Sequence data were compared with available National Centre for Biotechnology Information (NCBI) Genbank entries using the Basic Local Alignment Search Tool (BLAST) search function to assess evidence for variation within the British

T. gallinae isolates and their relationship with other published Trichomonadidae isolates (Table 8.1).

Table 8.1: Genbank entries used in the phylogeny based on the ITS1/5.8S rRNA/ITS2 region.

Species	Host	Genbank Accession Number
Trichomonas gallinae	Mourning dove *Zenaida macroura*	EU215369
Trichomonas gallinae	Pigeon (-)	AY349182
Trichomonas gallinae	Mauritian columbid	EF208019
Trichomonas gallinae	Rock pigeon *Columbia livia*	EU215364
Trichomonas gallinae	Collared dove *Streptopelia decaocto*	EU215363
Trichomonas gallinae	Pigeon (-)	TGU86614
Trichomonas gallinae	House finch *Carpodacus mexicanus*	EU290649
Trichomonas gallinae	Scrub jay *Aphelocoma californica*	EU290649
Trichomonas gallinae	Broad-winged hawk *Buteo platypterus*	EU215368
Trichomonas sp.	Mockingbird *Mimus polyglottos*	EU290650
Trichomonas vaginalis	Human *Homo sapiens*	AY349185
Trichomonas canistomae	Dog *Canis familiaris*	AJ784786.1
Tritrichomonas foetus	Cattle (-)	AY485679
Tetratrichomonas gallinarum	-	AY349181
Tetratrichomonas sp	Red-footed tortoise *Geochelone carbonaria*	AY886826
Pentatrichomonas hominis	Dog *Canis familiaris*	AY758392
Pentatrichomonas hominis	Human *Homo sapiens*	PHU86616
Ditrichomonas honigbergii	Free-living	AY349188
Trichomitus batrachorum	Snake (-)	AY349193
Hypotrichomonas acosta	Snake (-)	AY349192

DNA extracts were selected from the range of British birds (n=67) in which trichomonosis was diagnosed including Fringillidae (n=46), Columbidae (n=10), Accipitridae (n=3), Passeridae (n=2), Paridae (n=2), Prunellidae (n=2), Strigidae (n=1) and Turdidae (n=1). Fringillidae cases were selected from across Great Britain, to explore geographical variation, with temporal spread between emergence of the disease in British finches in 2005 and during the subsequent years when epidemic mortality occurred (2006-2007). The set of greenfinch and chaffinch DNA extracts included cases that had died at sites with high finch mortality (> 20 dead birds at 1 site), presumably corresponding to the most virulent strains of the parasite if strain variation was present,

and from sites where small numbers of dead finches were found (2-5 per site). DNA extracts were available from 2 wood pigeon cases of trichomonosis that occurred in 2002, before the emergence of trichomonosis in British finches. The reference strain *T. gallinae* (Rivolta) Stabler (ATCC Number 30230) and a *Trichomonad* sp. parasite cultured from the caecal contents of a Scottish semi-domesticated pheasant (supplied by Tom Pennycott, Scottish Agricultural College) were also amplified and sequenced using this PCR.

Nested PCR for the trichomonad SSU rRNA gene

A nested PCR was used to amplify the coding SSU rRNA gene following the protocol (Chapter 6). Sequences were compared with available gene sequences within the NCBI Genbank using the BLAST search function (Table 8.2).

Table 8.2: Genbank entries used in the phylogeny based on the SSU rRNA ribosomal gene.

Species	Host	Genbank Accession Number
Trichomonas gallinae	Broad-winged hawk *Buteo platypterus*	EU215375
Trichomonas gallinae	Collared dove *Streptopelia decaocto*	EU215374
Trichomonas gallinae	Coopers hawk *Accipiter cooperii*	EU215372
Trichomonas gallinae	Rock pigeon *Columba livia*	EU215373
Trichomonas equibuccalis	Horse *Equus caballus*	AY247750
Trichomonas tenax	-	U37711
Trichomonas vaginalis	Human *Homo sapiens*	AY338476
Trichomonas sp.	Common ground dove *Columbina passerina*	EU215371
Trichomonas sp.	White-winged dove *Zenaida asiatica*	EU215370
Tetratrichomonas gallinarum	Turkey *Meleagris galloparvo*	AJ920324
Monocercomonas columbrorum	Cuban Lizard (species unidentified)	DQ174303

DNA extracts were selected from the range of British birds (n=22) in which trichomonosis was diagnosed including Fringillidae (n=9), Columbidae (n=8), Prunellidae (n=1), Accipitridae (n=3) and Strigidae (n=1). Greenfinch cases were selected to include temporal spread between 2005 and 2007. DNA extracts were amplified and sequenced from 2 wood pigeon cases of trichomonosis that occurred in

2002, before the emergence of trichomonosis in British finches. The reference strain *T. gallinae* (Rivolta) Stabler (ATCC Number 30230) was also amplified and sequenced.

Fe-hydrogenase PCR

PCR was used to amplify the hydrogenosomal Fe-hydrogenase gene using TrichhydFOR (GTTTGGGATGGCCTCAGAAT) and TrichhydREV (AGCCGAAGATGTTGTCGAAT). The primers were designed using Primer-BLAST Primer Designing Tool software based on AF446077.1 (*Trichomonas gallinae* Fe-hydrogenase gene, partial cds from Voncken et al., 2002). Oligonucleotide primers were supplied by Operon Biotechnologies, Germany. PCR reactions were used with 5 µL of 10X PCR buffer (Qiagen, UK), 3 µL of 25mM $MgCl_2$ (Qiagen, UK), 0.5 µL of 5 U/µL HotStar Taq Plus DNA Polymerase (Qiagen, UK), 2 µL template DNA, 0.4 µL of 100mM dNTP mix (Bioline, UK), 3 µL of 10 µM forward and reverse primer and molecular grade water to complete the 50 µL per reaction. After an initial 15 min denaturation at 94 °C, 35 cycles of 94 °C for 1 min, 53°C for 30 sec and 72 °C for 1 min were carried out, followed by a 5 min extension at 72 °C using a thermal cycler (Geneamp PCR System 9700). Each amplification contained a negative control of water and a positive control of purified trichomonad DNA obtained from parasites cultured from an affected greenfinch.

The amplified PCR products were visualised under UV light after ethidium bromide staining of a 1% agarose gel and the expected product size (c. 1000 bps) was confirmed using Ready-Load 100bp DNA ladder (Invitrogen, UK). PCR products were cleaned using the QIAQuick gel extraction kit (Qiagen, UK) and submitted for sequencing at Cogenics (UK) using the ABI 3730 xl platform with the TrichhydFOR forward and TrichhydREV reverse primers. Chromatograph profiles were inspected using Chromas 2 software (www.synthesisgene.com). The sequence from the forward TrichhydFOR primer and the reverse complement of the TrichhydREV primer PCR product were aligned in both directions for each sample using Molecular Evolutionary Genetics Analysis (MEGA) 4.1 software and ClustalW (www.megasoftware.net). Sequences were compared with the only available Trichomonadidae gene sequences (*T. gallinae*

AF446077 and *T. vaginalis* from humans XM_001310179) within NCBI Genbank using the BLAST search function.

This study is the first to sequence the Fe-hydrogenase gene of multiple *T. gallinae* parasites, consequently, other than a single sequence, no comparable data were available within Genbank to evaluate strain variation. To provide an outgroup set of data for the British bird isolates, the gene from *T. gallinae* isolates from columbid species from the Seychelles was also amplified and sequenced. Samples were collected in 2007 from the Seychelles blue pigeon (*Alectroenas pulcherrima* n=4), the Madagascar turtle dove (*Streptopelia picturata* n=2) and the zebra dove (*Geopelia striata* n=1). Oropharyngeal swabs from live columbids were inoculated in to In-Pouch kits (BioMed Diagnostics) and incubated at 30 °C. Positive cultures were archived in 100% ethanol and stored at 4 °C prior to extraction using the same technique.

Phylogeny construction

The evolutionary history for each of the phylogenies was inferred using the Neighbour-Joining method (Saitou et al., 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1025 replicates) was calculated (Felsenstein 1985). The trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. Phylogenetic analyses were conducted in MEGA 4 (Tamura et al., 2007).

Random Amplified Polymorphic DNA (RAPD) analysis

A library of trichomonad parasites collected from culture of lesions collected at post mortem examination was cryopreserved at the Institute of Zoology. Crop lesions from cases with suspected trichomonosis were incubated at 30°C in Oxoid Trichomonas Media No. 2. (Oxoid, UK) and screened for motile trichomonads at 24, 48, 72 hrs and 5 days. Cultures of motile trichomonads in Oxoid Trichomonas Media No. 2. (Oxoid, UK) were screened for evidence of fungal contamination by examining wet preparations

under the light microscope. Amphotericin B (Fungizone 250 µg/ml; Gibco, UK) was used (0.2 ml per 5 ml of culture media) where required to remove fungal contamination. Pure trichomonad cultures with high parasite motility were selected for cryopreservation. Parasites were gently pelleted from 1 ml of culture media by centrifugation at 2,000 rpm for 1 minute and then re-suspended in Phosphate Saline Glucose (PSG) (488.8mg/l NaH2PO4, 2.55g/l NaCL, 8.08g/l Na2HPO4, 15g/l D-glucose) plus 14% glycerol. A Nalgene 1 Cryogenic Freezer Container (U.S.A.) was used for slow cooling to -80 °C, with transfer to -140 °C/ liquid nitrogen in some cases. Samples were raised from the archive library, rapidly thawed at 30 °C and propagated in culture by repeating the above protocol. DNA extraction was performed on live trichomonad cultures with high motility, free from fungal contamination, and were stored at 4 °C. The DNA concentration was measured using a Nanodrop (Thermo Scientific, U.S.A.) and samples with DNA concentrations greater or equal to 10 ng/µl, with good quality based on an acceptable A260:280 and A260:230 ratio, were selected for RAPD analysis. DNA extracts were selected for RAPD analysis from the range of British birds (n=14) in which trichomonosis was diagnosed, where available, including Accipitridae (sparrowhawk *Accipiter nisus* n=1), Columbidae (collared dove *Streptopelia decaoacto* n=1, wood pigeon *Columba palumbus* n=1), Fringillidae (greenfinch n=4, chaffinch n=2, brambling *Fringilla montifringilla* n=1 goldfinch *Carduelis carduelis* n=1), Paridae (great tit *Parus major* n=1), Prunellidae (dunnock *Prunella modularis* n=1) and the reference strain *T. gallinae* (Rivolta) Stabler (ATCC Number 30230).

5'[6_FAM]-labelled OPD primers (OPD3, OPD5, OPD7 and OPD8), supplied by Sigma Aldrich, UK, were used in an adapted protocol from Felleisen (1998) and Gaspar da Silva et al. (2007). PCR reactions were used with 5 µL of 10X PCR buffer (Qiagen, UK), 3 µL of 25mM $MgCl_2$ (Qiagen, UK), 0.5 µL of 5 U/µL HotStar Taq Plus DNA Polymerase (Qiagen, UK), 25ng genomic template DNA, 0.4 µL of 100mM dNTP mix (Bioline, UK), 3 µL of 10 µM OPD primer and molecular grade water to complete the 50 µL per reaction. After an initial 15 min denaturation at 94 °C, 40 cycles of 94 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min were carried out, followed by a 15 min

extension at 72 °C using a thermal cycler (Geneamp PCR System 9700). Each amplification contained a negative control of water and a positive control of purified trichomonad DNA obtained from parasites cultured from an affected greenfinch.

The RAPD analysis was repeated in triplicate. The amplified PCR products were visualised under UV light after ethidium bromide staining of a 2% agarose gel and the product sizes were determined using Ready-Load 100 bp DNA ladder (Invitrogen, UK). The gel band profiles from each replicate, for each OPD primer, were compared visually to verify that the results were highly reproducible.

PCR products from 2 replicates were submitted to the John Innes Genome Laboratory where they were diluted 1 in 30 with molecular grade water. 1 µl of the diluted PCR product was added to 8.9 µl of HiDi Formamide (Applied Biosystems 4311320) and 0.1 µl of ROX Ladder (Web Scientific MRK1000) for a total of 10 µl per sample. Samples were loaded directly onto an Applied Biosystems 3730xl DNA Analyzer without heat denaturation. Samples were run with Dye set D on a 50cm array with Pop7 using a Run Voltage of 8.0V and Run Time of 6000 sec with all other settings set to default values. The .fsa file outputs from the 3730xl DNA Analyzer were imported into GeneMarker software, version 1.90 beta (SoftGenetics LLC, U.S.A.) and calibrated against the ROX Ladder x1000 to determine the fragment size for each peak representing the genotype of the sample. A panel was constructed for each OPD primer set. The panel consisted of all the alleles for all the genotypes from all the samples for that primer set. The sample data files were analyzed using the panel, recording the presence or absence of each peak, or allele of the genotype, and expressed in binary matrix format. The size of bands assessed was restricted within the range of the ROX 1000 Ladder. The band profiles from both replicates were manually examined and verified independently for each OPD primer, to remove inconsistencies or artifacts, and compared to confirm high reproducibility of results; only bands that were present in both replicates were included in the RAPD analyses. Clustering analyses were performed to generate a dendrogram for each of the 4 OPD

primer sets in isolation, expressed on the basis of percentage similarity. The GeneMarker merge project tool was then used to perform a meta-analysis of data combined from all 4, individual OPD primer sets.

Where sample availability and quality permitted, DNA isolates were used from the same birds for both the Fe-hydrogenase PCR and RAPD analyses to enable direct comparison (Table 8.3).

Table 8.3: Cases from which DNA isolates were used in the Fe-hydrogenase PCR and RAPD analyses.

Case number	Species	Year	Fe-hydrogenase	RAPD
62-09	**Brambling**	2009	Yes	Yes
B1514	Bullfinch	2007	Yes	No
820-07	Chaffinch	2007	Yes	No
796-08	Chaffinch	2008	No	Yes
861-08	**Chaffinch**	2008	Yes	Yes
867-08	Chaffinch	2008	Yes	No
963-05	Collared dove	2005	Yes	No
811-07	**Collared dove**	2007	Yes	Yes
528-07	Common buzzard	2007	Yes	No
815-07	Dunnock	2007	No	Yes
89-08	Goldfinch	2008	No	Yes
1036-05	Great tit	2005	Yes	No
1035-07	**Great tit**	2007	Yes	Yes
760-07	Greenfinch	2007	Yes	No
810-07	Greenfinch	2007	No	Yes
837-07	Greenfinch	2007	Yes	No
892-07	Greenfinch	2007	No	Yes
90-08	Greenfinch	2008	Yes	No
99-08	Greenfinch	2008	Yes	No
858-08	Greenfinch	2008	Yes	No
864-08	**Greenfinch**	2008	Yes	Yes
R2003	Greenfinch	2008	No	Yes
R2056	Greenfinch	2008	No	Yes
1020-07	**Sparrowhawk**	2007	Yes	Yes
484-07	Wood pigeon	2007	Yes	No
1337-07	Wood pigeon	2007	No	Yes

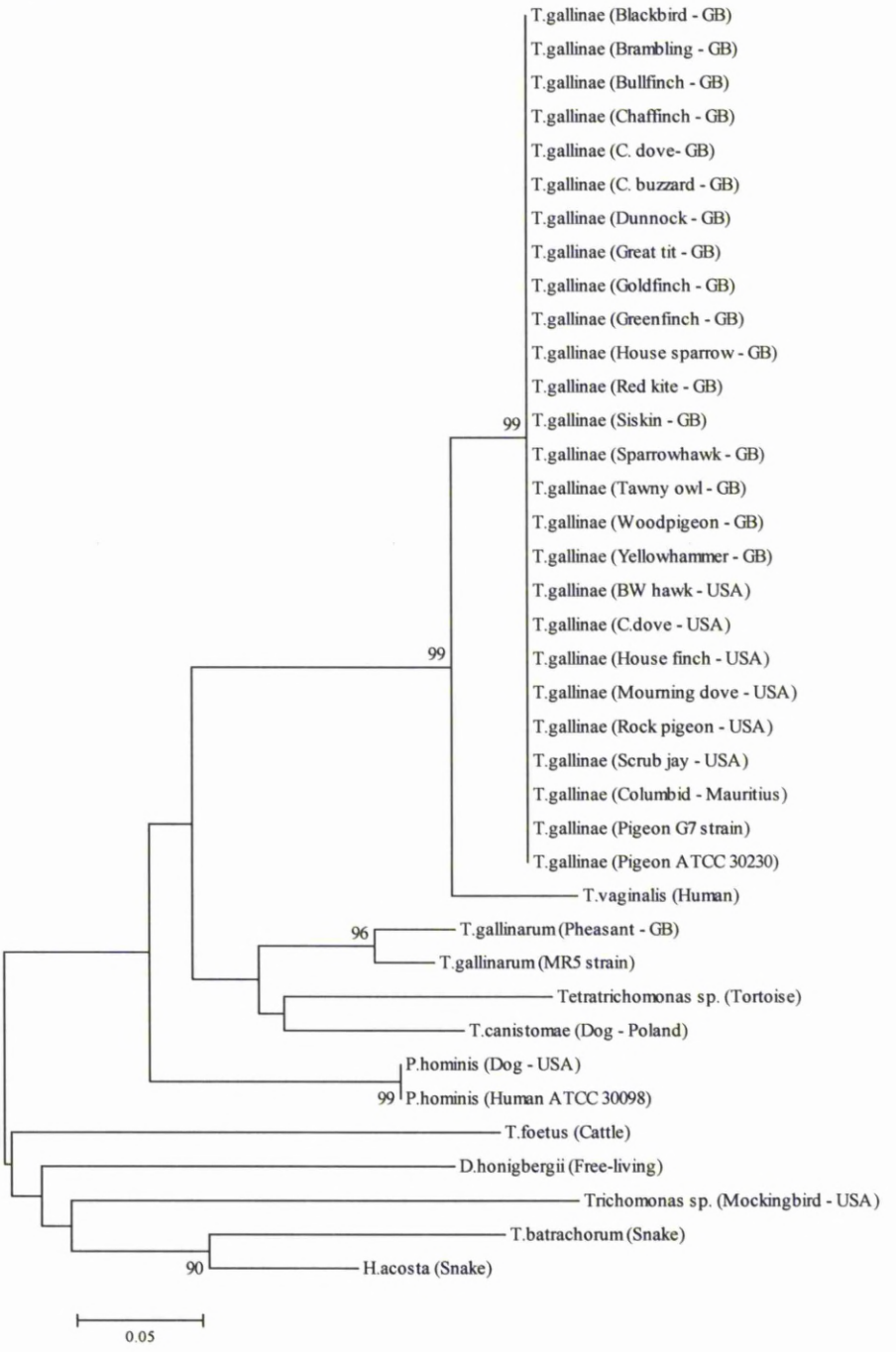
*Samples marked in bold have results available for both Fe-hydrogenase PCR and RAPD analysis.

8.3 RESULTS

PCR for the ITS1/ 5.8S rRNA/ ITS2 region

An identical nucleotide sequence (214 nucleotides) was obtained from all the British bird cases following amplification of the ITS1/5.8S rRNA/ITS2 region. No sequence variation was detected between the British isolates according to species, geographical region or year (Figure 8.1). Sequence data from columbiform isolates collected before and following emergence of trichomonosis in finches were identical. The reference strain *T. gallinae* (Rivolta) Stabler (ATCC Number 30230) differed from the British isolates with 3 point mutations and 2 deletions. The Scottish pheasant sample generated a divergent sequence contrasting with those derived from samples from other wild birds in Great Britain, differing with 47 point mutations and 3 insertions from the finch and columbiform samples. A Genbank NCBI BLAST search confirmed that the pheasant sequence clustered with 99% maximum identity to a *Tetratrichomonas gallinarum* (AY245129) sequence.

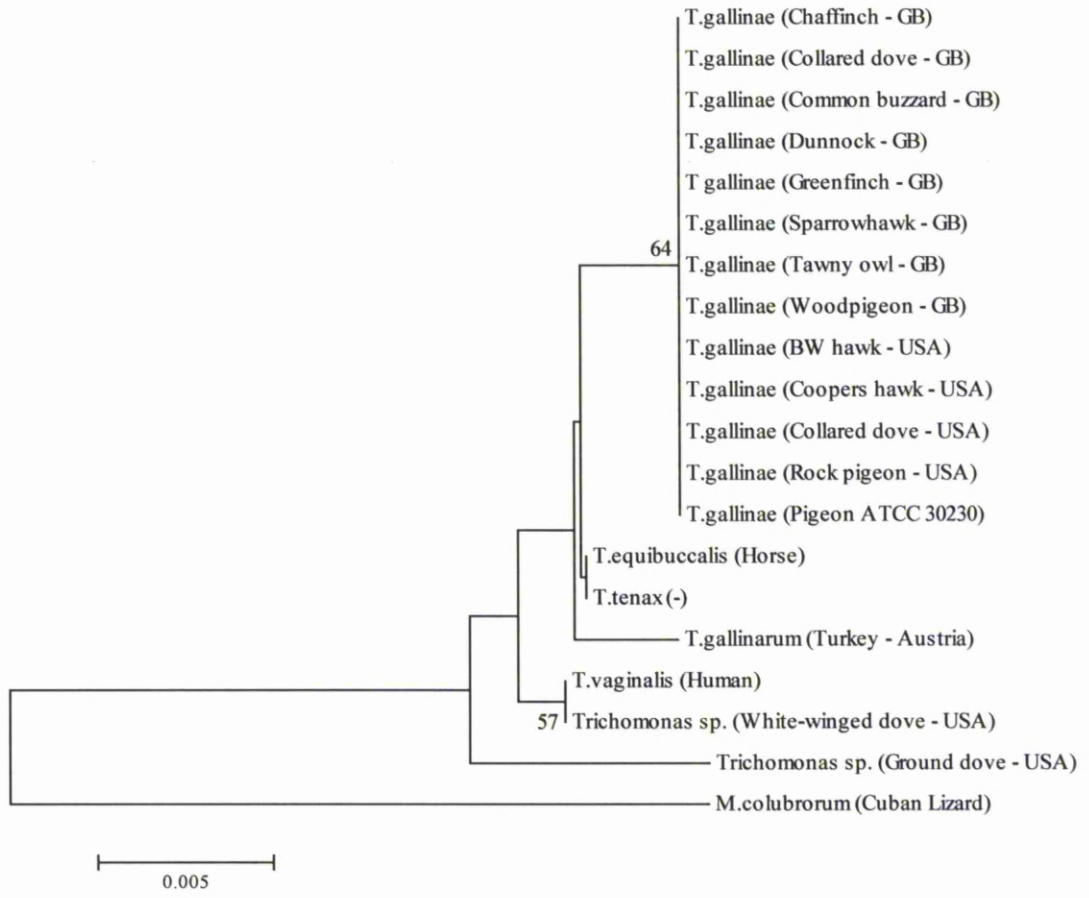
Figure 8.1: Phylogeny based on the ITS1/ 5.8S rRNA / ITS2 region constructed using the Neighbour-Joining method.
The evolutionary history of 38 taxa was inferred using the Neighbour-Joining method. There were a total of 184 positions in the final dataset. The optimal tree with the sum of branch length = 1.49853447 is shown.



Nested PCR for the SSU rRNA gene

An identical nucleotide sequence (149 nucleotides) was obtained from all the British bird cases that were screened by the nested PCR with trichomonad SSU rRNA primers followed by TN3 and TN4 nested primers. No sequence variation was detected between the British isolates according to species, geographical region or year (Figure 8.2). Sequence data from columbiform isolates collected before and following emergence of trichomonosis in finches were identical. Sequence data from the reference strain *T. gallinae* (Rivolta) Stabler (ATCC Number 30230) was also identical.

Figure 8.2: Phylogeny based on the SSU rRNA gene constructed using the Neighbour-Joining method. There were a total of 149 positions in the final dataset. The optimal tree with the sum of branch length 0.05045443 is shown.



Fe-hydrogenase PCR

High quality nucleotide sequence of variable length (930 - 999 nucleotides) was obtained from the British bird cases following amplification of the Fe-hydrogenase gene. A Neighbour-Joining phylogenetic tree was constructed from a 903 nucleotide sequence available from all samples (Figure 8.3). All clusters were supported by high bootstrap values. No sequence variation was detected between the British isolates according to species, geographical region or year. Multiple attempts to amplify this gene from the 2002 columbiform isolates were unsuccessful. Marked divergence in the sequence data was found with 18 point nucleotide substitutions between the British isolates, the *T. gallinae* (Rivolta) Stabler (ATCC Number 30230) reference strain and the single *T. gallinae* isolate available within Genbank (AF446077) (Table 8.4). Sequence diversity was also observed between the *T. gallinae* isolates obtained from different columbid species on the Seychelles, indicating that a number of strains of *T. gallinae* are circulating in this island's avian fauna (Table 8.4).

Figure 8.3: Phylogeny based on the Fe-hydrogenase gene constructed using the Neighbour-Joining method. There were a total of 149 positions in the final dataset. The optimal tree with the sum of branch length =0.20417397 is shown.

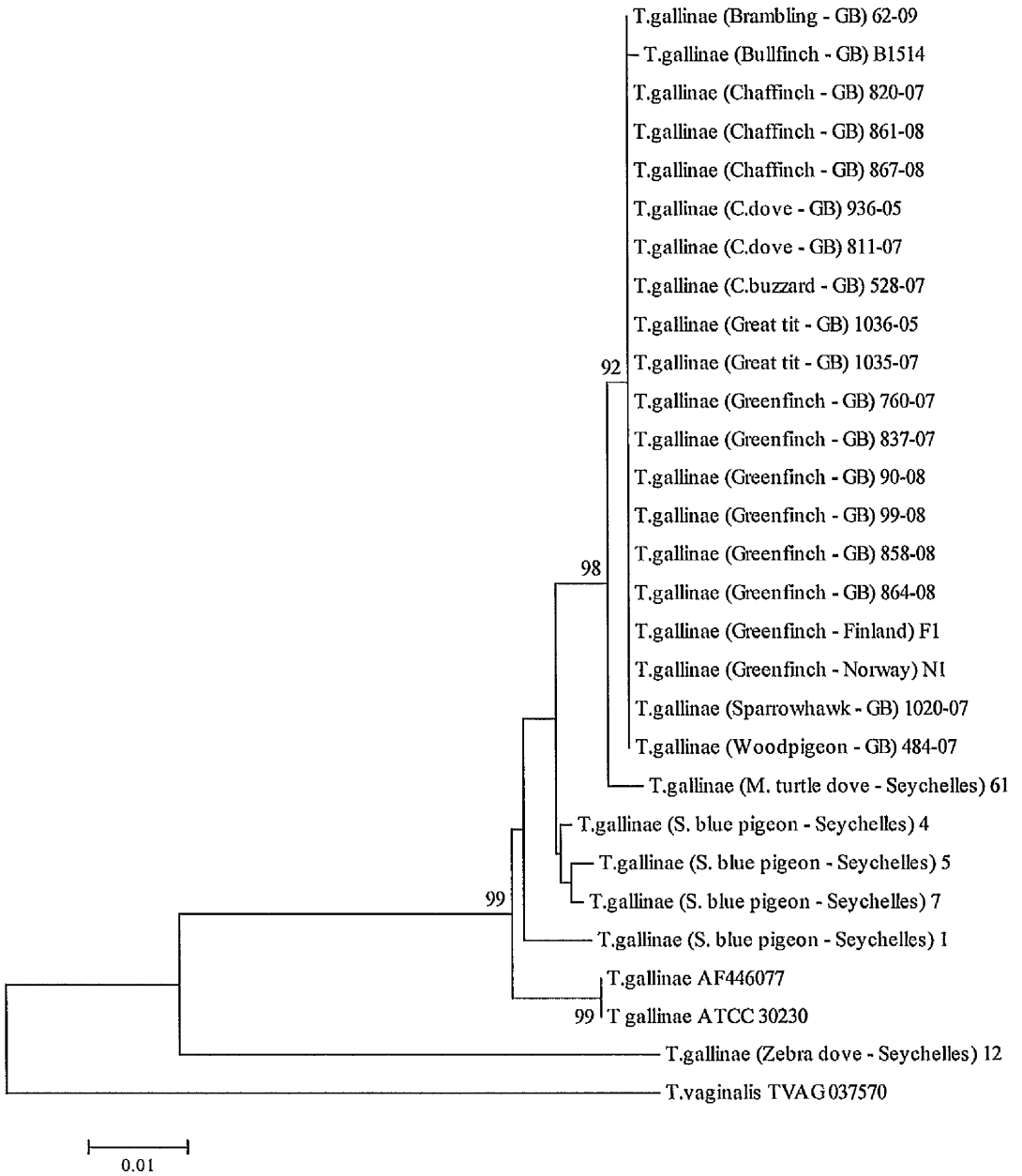


Table 8.4: Genetic differences (number and percentage of base pair polymorphisms) between the sequence data for the Fe-hydrogenase gene (903 bp sequence) from *T. gallinae* collected from British bird species (used as baseline), the Seychelles columbid and the Genbank Trichomonadidae entries.

Sample	Number of substitutions	Number of insertions	Number of deletions
T. gallinae Great Britain bird species	0	0	0
T. vaginalis TVAG_037570	103 (11%)	0	0
T. gallinae AF446077	18 (2%)	0	0
T. gallinae ATCC 30230	18 (2%)	0	0
T. gallinae (S. Blue pigeon - Seychelles) 1	16 (1.8%)	0	0
T. gallinae (S. Blue pigeon - Seychelles) 4	8 (0.9%)	0	0
T. gallinae (S. Blue pigeon - Seychelles) 5	10 (1.1%)	0	0
T. gallinae (S. Blue pigeon - Seychelles) 7	9 (1.0%)	0	0
T. gallinae (M. turtle dove - Seychelles) 61	5 (0.6%)	0	0
T. gallinae (Zebra dove - Seychelles) 12	77 (8.5%)	1 (0.1%)	0

RAPD

Visual comparison of the band profiles on the agarose gels for each of the 4 OPD primer sets indicated high reproducibility between the 3 sets. Modification of the RAPD protocol, utilising 5'[6_FAM]-labelled primers with automated reading of gel profiles was successful; again high reproducibility was achieved between the 2 sets. Data were incorporated from 24 – 62 bands of size between 92 - 999 bp for the 4 OPD primer sets.

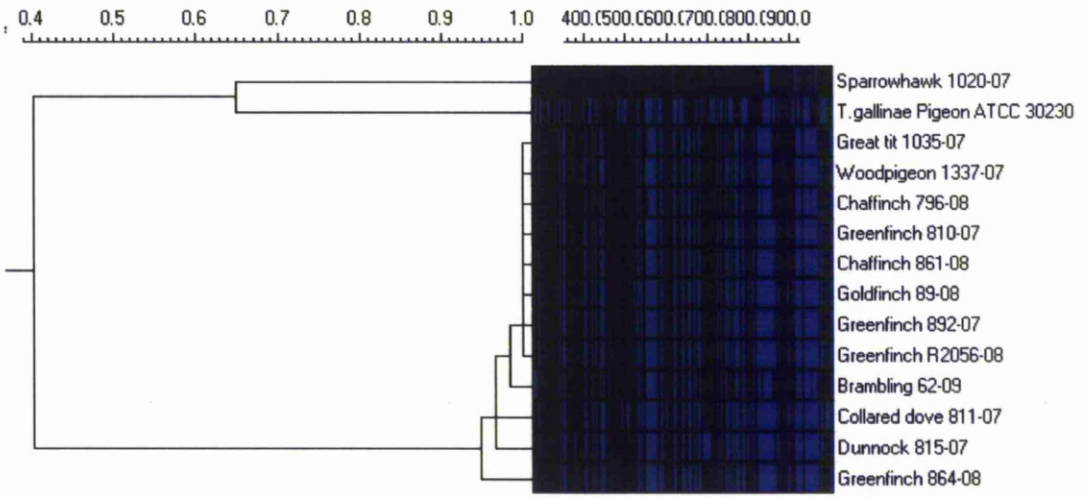
Some variation was observed between the results of the 4 OPD primers (Figure 8.4). Three of the OPD primer sets support minimal variation between the British passeriform and columbiform isolates with percentage similarity between isolates >95% for OPD3 and OPD5, and >85% percentage similarity for OPD8. For OPD7, 9 of the British passeriform and columbiform isolates had >90% percentage similarity, however, the other 3 isolates gave more divergent results. Each of the OPD primer sets supported a clonal origin for the British passeriform and columbiform isolates.

The *T. gallinae* (Rivolta) Stabler (ATCC Number 30230) reference strain was most divergent and this was consistent for each of the OPD primers sets. The sparrowhawk isolate from England collected in 2007 was also highly divergent: this isolate clustered

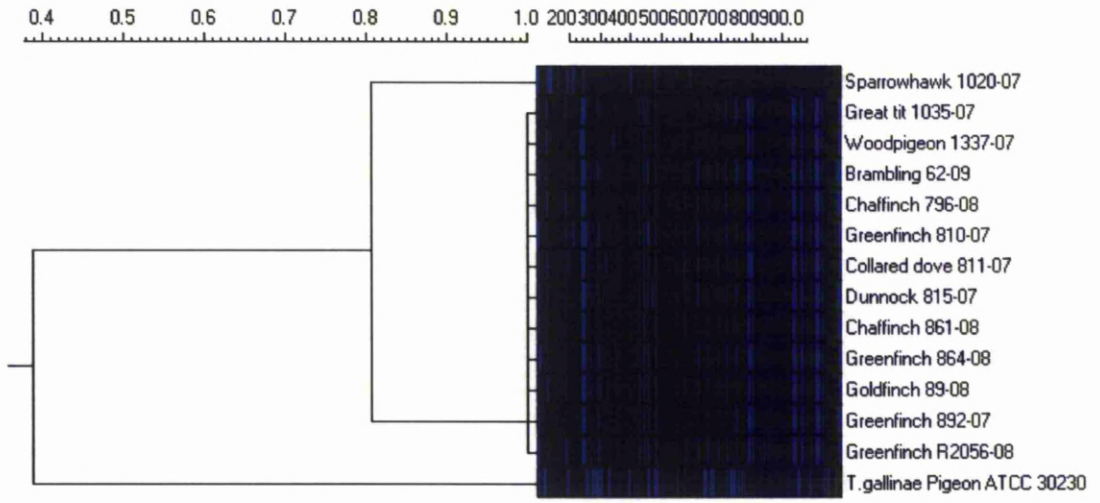
with the *T. gallinae* reference strain for OPD3 and OPD7 primer sets, and clustered with the passeriform and columbiform isolates for the OPD5 and OPD8 primer sets.

Figure 8.4: RAPD dendrogram for each of the 4 OPD primer sets.
(a) OPD3 (b) OPD5 (c) OPD7 (d) OPD8.
Left scale denotes percentage similarity between isolates.
Right scale denoted band size bp.

(a)

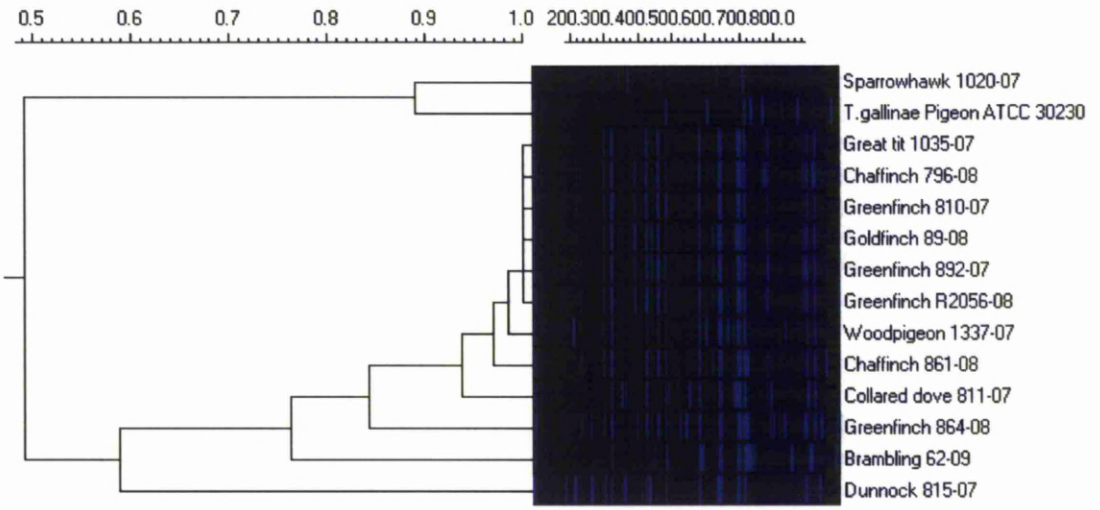


(a)

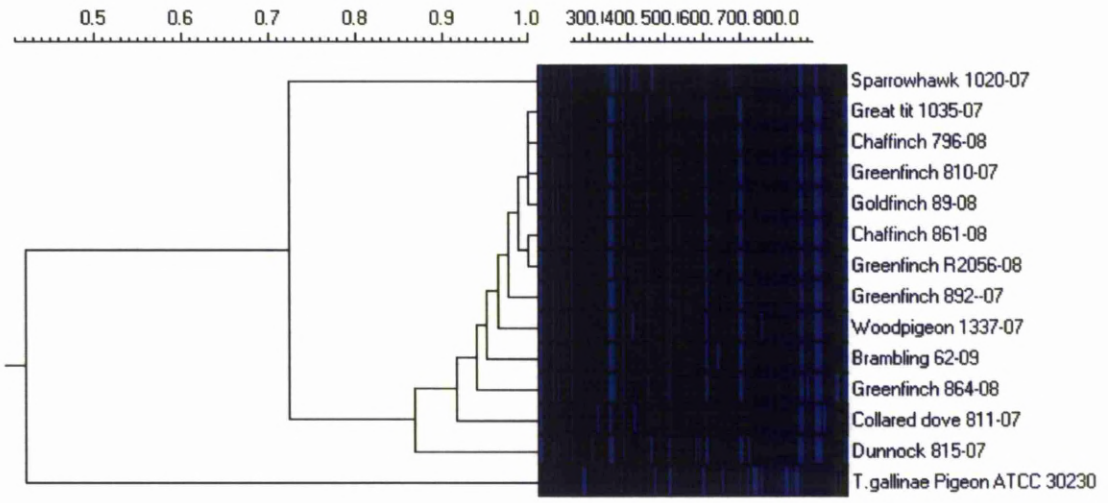


Left scale denotes percentage similarity between isolates.
Right scale denoted band size bp.

(c)

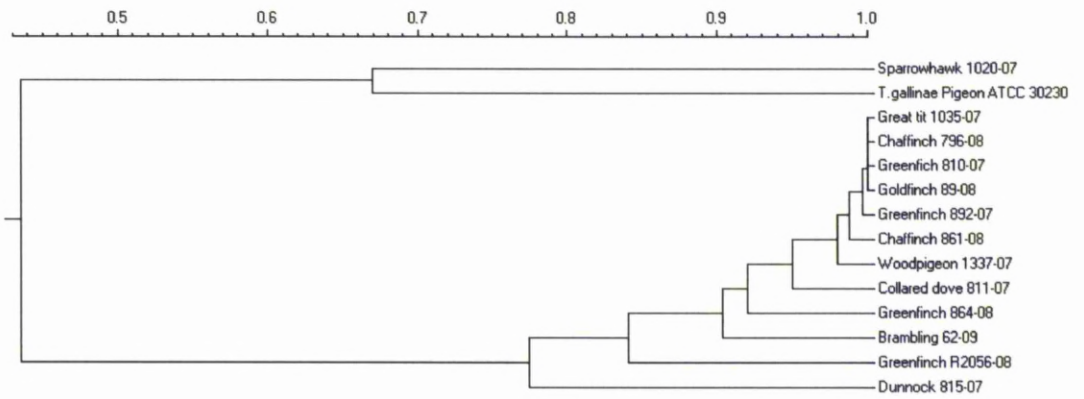


(d)



The meta-analysis incorporating data from each of the OPD primers produced a summary dendrogram (Figure 8.5) which supports the clonal origin for the British Passeriform and Columbiform isolates.

Figure 8.5: RAPD dendrogram for the OPD primer sets in combination. Scale denotes similarity between isolates expressed as a proportion.



8.4 DISCUSSION

Examination of sequence data from the ITS 1/ 5.8S rRNA/ ITS2 region, a partial fragment of the trichomonad SSU rRNA gene and the Fe-hydrogenase gene found no evidence of genotypic variation between the *T. gallinae* strains examined from British garden birds. All isolates from the greenfinch and chaffinch, the species most frequently affected by the disease, were identical suggesting that a clonal strain was responsible for the outbreak, as compared with multiple strains identified in some previous epidemics (Gerhold et al., 2008). No change in the sequence data was observed over time (2005 – 2007) and no variation according to region across Great Britain was observed. *T. gallinae* isolates from finches collected from sites with high levels of mortality (>20 dead birds) had the same sequence data as all other isolates, consequently, there was no evidence for genotypic strain variation influencing virulence.

No sequence variation was found between *T. gallinae* isolates cultured from finch, columbiform or raptor species supporting the hypothesis that a common strain, rather than multiple strains, was circulating in the British avian fauna subsequent to 2005. Predatory raptor species could have become infected through predation on sick columbiform or finch prey, whilst scavenging raptor species could become infected through feeding on recently dead carrion.

Sequence data from the Scottish pheasant sample at the ITS 1/ 5.8S rRNA/ ITS2 region was clearly distinct from the parasite strain causing mortality in British finches and consistent with *Tetratrichomonas gallinarum*, an intestinal parasite of galliform and anseriform species. *Trichomonas phasani* has been reported as an intestinal parasite, inconsistently associated with enteritis, in Scottish pheasants (Higgins 1980; Pennycott 1998). This species identification relied on morphology of the trichomonad parasite and the avian host taxa. This is the first time that molecular techniques have been employed for the species identification of *Trichomonas phasani*. Further research from a larger number of samples is required to determine whether *Trichomonas phasani* and *Tetratrichomonas gallinarum* are indeed synonymous. Whilst only a single sequence was available from the ITS 1/ 5.8S rRNA/ ITS2 region of a trichomonad parasite from a British pheasant, these divergent sequence results, coupled with the contrasting anatomical sites from which the parasites were cultured (caecal contents in pheasant versus oesophagus in finches) and pathology (enteritis versus necrotic ingluvitis, although Pennycott (1998) recovered trichomonad parasites from the upper alimentary tract of a minority of pheasants in his study) indicate that game birds are an unlikely origin of the *T. gallinae* strain in British finches.

Unfortunately, relatively few columbiform isolates predating the emergence of trichomonosis in finches in Britain were available for study and, due to sample degradation, sequence data for the 2002 columbiform isolates could only be obtained for the ITS 1/ 5.8S rRNA/ ITS2 region and SSU rRNA gene. No variation was found between the *T. gallinae* isolates obtained from columbiform species before the outbreak, and those from finches and columbiform species collected following emergence of trichomonosis in finches. These results are consistent with the hypothesis that the

epidemic in finches occurred due to spill-over of an existing *T. gallinae* strain from sympatric British columbiform species. However, with the limited sample number of columbiform isolates available from pre-2005, coupled with the absence of Fe-hydrogenase gene sequence data or RAPD analyses from the two 2002 cases due to poor sample quality, it may be that it is not possible to fully appreciate genotypic variation that was present in the British bird population prior to 2005 and definitive conclusions on the origin of the *T. gallinae* strain affecting British finches are not possible.

Centres with an interest in wild bird disease (Institute of Zoology, Scottish Agricultural College, Wildlife Veterinary Investigation Centre, *unpublished data*; C. Davis *pers. comm.*) across Great Britain have received anecdotal reports of columbiform morbidity and mortality due to trichomonosis since the mid-nineties, through members of the public or those involved in game bird rearing, and investigated some of the incidents. Cousquer (2003) reviewed retrospective cases of trichomoniasis in predatory and prey bird species submitted to a southwest wildlife hospital from 1998 to 2002. He noted an increased annual incidence of trichomoniasis in 2000, affecting 13% (30/226) of wood pigeon casualties and 7% (11/166) of collared dove casualties as compared with 4-8% and 2-4% of the submissions, for each of the species respectively, for the other study years. Duff (2002) reported significant mortality of wood pigeons and collared doves throughout southern England during autumn 2002 caused by trichomonosis, although no estimate of the scale of mortality was available. In 2005, V. Simpson (*pers. comm.*) investigated an outbreak of trichomonosis affecting wood pigeons in Somerset with high reported mortality rates. Höfle et al. (2004) reported an outbreak of trichomoniasis in wood pigeons in southern Spain in 2001; supplementary feeding of game birds influencing bird congregation was considered an important risk factor for this outbreak. The Veterinary Investigation Surveillance Report (VIDA), published since the 1990s by the Veterinary Laboratories Agency, includes sporadic single figure diagnoses of trichomonosis in wood pigeons in most years with wide geographical coverage and no clear temporal pattern: this surveillance dataset shows no evidence to support a large-scale disease event in columbiform species in the years preceding emergence of finch trichomonosis.

Collectively, these findings indicate that regional outbreaks of columbiform trichomonosis occurred in the 5-year period preceding the first diagnosis of finch trichomonosis. These reports are *ad hoc* in nature with minimal information available on the scale of mortality, temporal or spatial extent of the outbreaks: no systematic surveillance scheme is available to provide a historical baseline against which to compare these events. Consequently, it is not possible to conclude whether or not these events provide strong evidence to support a major change in the epidemiology of trichomonosis in columbiform species in Great Britain, which might indicate emergence of a novel virulent strain of the parasite before 2005, when the disease in finches was first recognised. In the absence of an alternative avian host origin, the explanation of parasite spill-over from columbiform species to sympatric finches appears most plausible.

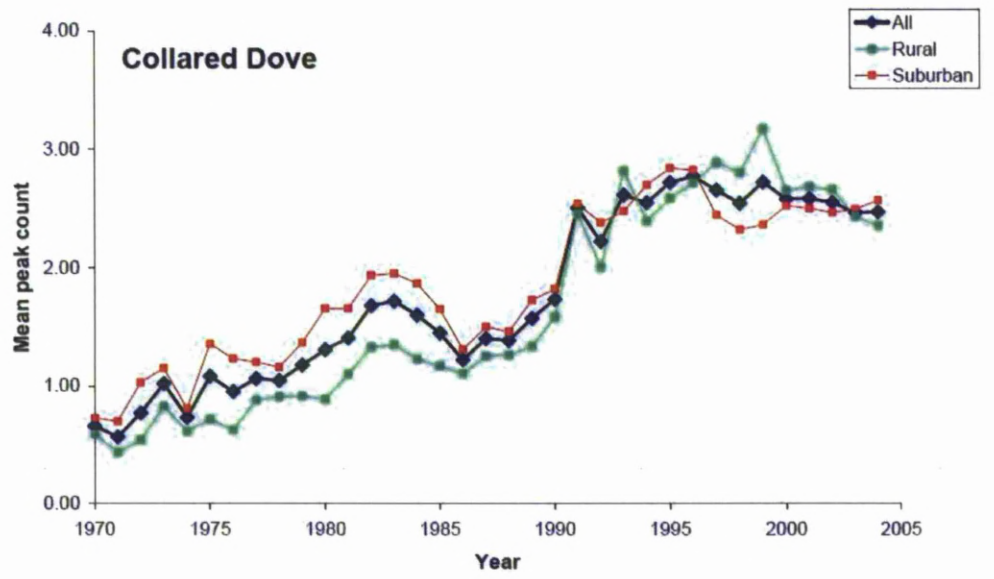
Anderson et al. (2009) investigated the molecular epidemiology of endemic, rather than epidemic as in British finches, trichomonosis infection in northern Californian passerine species and found the strain to be identical to sympatric free-ranging columbiform species and raptors, as in the current study. These authors highlighted communal feeding and water sources as a potential route for spread and suggested that localised spill-over from infected columbiforms to house finches occurred at these sites. Shared food and water sources at supplementary feeding stations were also put forward as the likely mechanism for spread of trichomonosis resulting in an epidemic of disease in Spanish wood pigeons (*Columba palumbus*) (Villanúa et al., 2006). *T. gallinae* spread to passerine species of other families, for example the Prunellidae, Passeridae, Prunellidae and Turdidae, is considered most likely to have occurred from finches through shared food and water sources at garden feeding stations.

Feeding of garden birds is a popular pastime with the British public which may provide opportunities for species mixing and congregation at feeding stations. The BTO Garden Bird Feeding Survey (Chamberlain et al., 2005) data indicate a marked increase in the peak count index of columbiform species, particularly of wood pigeons over the past decade (Figure 8.6). This upward trend in wood pigeons is attributed to the intensification of arable cultivation, which promotes increased overwinter survival,

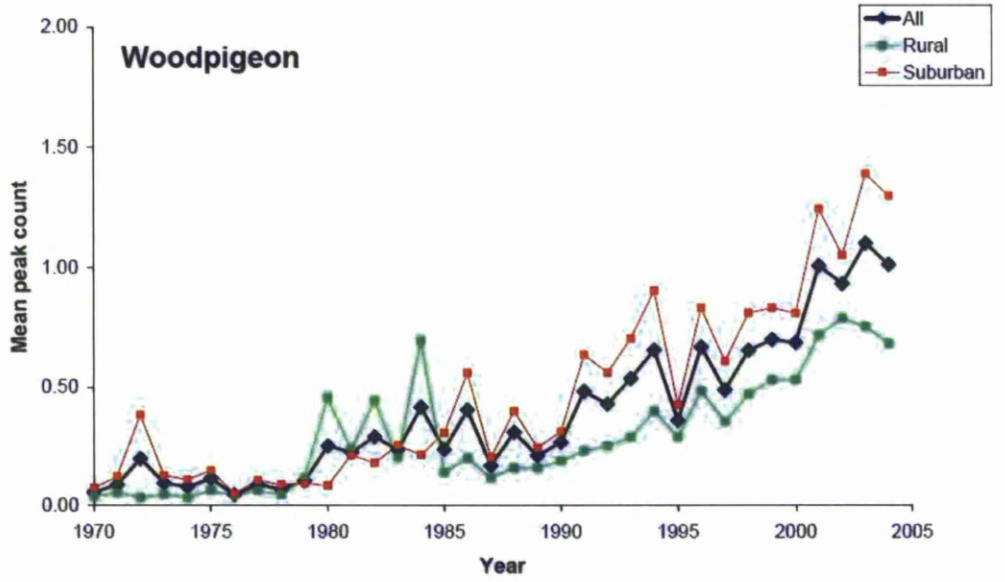
resulting in an increased population size and spill-over from farmland to garden habitats (Gibbons et al., 1993). In turn this may have led to increased mixing of Fringillidae and Columbidae species in close proximity, providing opportunities for parasite spread.

Figure 8.6: BTO Garden Bird Feeding Survey peak count index for the (a) collared dove and (b) wood pigeon, 1970-2005. National data (blue line) are also shown split by garden habitat type (urban/suburban and rural).

(a)



(b)



Sequence data from the Fe-hydrogenase gene identified considerable variation in the *T. gallinae* isolates obtained from the 3 columbid species from the Seychelles and the *T. gallinae* (Rivolta) Stabler (ATCC Number 30230) reference strain. The Fe-hydrogenase gene offers a useful marker to investigate intra- and inter-specific strain variation within the Trichomonadidae in the future. The lack of sequence variation from this gene in the British *T. gallinae* isolates, when compared with the diversity observed in the Seychelles and reference strains, further supports the clonal origin of the parasite involved in the British finch trichomonosis epidemic.

The modified RAPD technique with automated gel reading employed in this study was successful and provides a tool to improve accuracy and reduce subjective and gel-gel variation associated with these analyses. The large ladder size (Rox Ladder x1000) enabled us to calibrate against, and interpret, bands of greater size (<1000 bp) as compared with smaller commercial ladders typically used in these applications. As with sequence analysis of the Fe-hydrogenase gene, the RAPD analyses indicate that the *T. gallinae* reference strain is divergent from the British bird isolates. In contrast to the sequence data, however, the RAPD analysis indicates that the single sparrowhawk isolate was distinct from the other British bird isolates. Due to the demands of DNA quality and purity required for RAPD analysis, the sample archive of *T. gallinae* isolated from raptors was limited and no columbiform samples predating emergence of the epidemic in finches were available. It is plausible that multiple parasite strains were circulating in British columbiforms, in turn infecting birds of prey, and it has not been possible to detect this in this study.

Whilst all the OPD primers indicated a clonal origin for the passeriform and columbiform isolates, the RAPDs showed fine-scale variation between passeriform isolates with no apparent temporal or spatial trends, or with host species. It is plausible that the RAPD analyses detected strain variation inapparent in the sequence data which could potentially be used for source tracking in the future However, the extent of variation between samples was relatively small making it most plausible that a single *T.*

gallinae strain was widespread and circulating in British finch and columbiform populations subsequent to the emergence in 2005.

RAPD analysis has several disadvantages when compared with phylogenetic analysis of sequence data. RAPD analysis requires high quality DNA extracted from live parasite culture, confirmed clear of fungal contamination which limited the number of isolates available in this study. All aspects of the methodology must be standardised as far as possible to reduce variation (e.g. sample collection, Taq enzyme source and lot, PCR machine, primer to template concentration ratio) which constrains use of this approach (Soll 2000). In comparison, PCR and sequencing was possible on parasite DNA extracted from lesions in birds examined post mortem which were insufficiently fresh for culture, consequently, a much larger sample size was available.

The results of this study indicate that sequence analysis of the Fe hydrogenase gene provides a superior genotyping marker to discriminate between *T. gallinae* strains, in comparison to the ITS1/ 5.8S rRNA/ ITS2 region and SSU rRNA gene which detect minimal variation. Sequence analyses methodologies are preferable to RAPD studies since the results are more robust and DNA sample requirements are less stringent.

Examination of *T. gallinae* strains from other host species and geographical regions is required to gauge existing sequence diversity and increase our understanding of the epidemiology of trichomonosis in wild bird populations. As finch trichomonosis spreads across continental Europe (Chapter 9), research is required to determine the extent to which this EID of finches will alter the global understanding of trichomonosis epidemiology in wild bird populations.

CHAPTER 9: THE INTERNATIONAL SPREAD OF TRICHOMONOSIS, AN EMERGING INFECTIOUS DISEASE, BY MIGRATING BIRDS

9.1 INTRODUCTION

The global spread of emerging infectious diseases (EIDs) can be facilitated by a variety of means and much attention has been given to anthropogenic factors such as habitat degradation and urbanisation, international travel, regulated and unregulated trade and species translocation (Daszak et al., 2001; Bradley et al., 2007; Gauthier-Clerc et al., 2007). Pathogen movement and disease spread also can occur through non-anthropogenic routes, for example by long-distance movement of migratory species, and this subject has been particularly topical in recent years following the emergence of H5N1 highly pathogenic avian influenza (HPAI) (Gilbert et al., 2006; Kilpatrick et al., 2006; Gauthier-Clerc et al., 2007; Keawcharoen et al., 2008).

Trichomonosis has been infrequently diagnosed in captive and free-ranging finches prior to 2005 (Chalmers 1992; NWHC 2002; Gerhold et al., 2008; Anderson et al., 2009). However, in 2005 it was recognised as an emerging infectious disease of wild finches in Great Britain (Pennycott et al., 2005a). Epidemic mortality of greenfinches (*Carduelis chloris*) and chaffinches (*Fringilla coelebs*) due to trichomonosis occurred in Britain in 2006, peaking in the autumn months (Lawson et al., 2006b). This is the first time that large-scale epidemic mortality in a non-columbiform species due to trichomonosis had been identified.

Although both greenfinch and chaffinch are widespread across Great Britain (Newson et al., 2008), peak trichomonosis-associated finch mortality occurred in the western and central counties of England and Wales in 2006, whilst the number of incidents observed in eastern England counties was low (Chapter 6). In this chapter, gene sequence data of *Trichomonas gallinae* strains is used to provide evidence that infections reported in Fennoscandian finches in 2008 (Neimanis et al., 2010) resulted from expansion of the range of the *T. gallinae* strain from Great Britain following its spread into the eastern

counties of England in 2007. Historical ring recovery data from long-term ornithological monitoring are explored to determine whether bird migration provides a plausible mechanism for the observed pattern of disease spread and, if so, which species are the most probable vectors.

9.2 MATERIALS AND METHODS

Spatial distribution of the epidemic in Great Britain

Opportunistic and systematic surveillance data for garden bird mortality was used to document the distribution of trichomonosis-associated mortality in 2006 and 2007 in Great Britain (Chapter 7). Trichomonosis incidents were defined according to specified criteria (Chapter 6). The number of trichomonosis incidents received through the opportunistic scheme was expressed per 1,000 households (according to the 2001 population census data (ONR 2005)) and the geographical distribution of the disease range was compared between years.

Ring return data

The British Trust for Ornithology operates the British and Irish Ringing Scheme[2] under which volunteers annually ring c. 800,000 birds a year (Balmer et al., 2008). Of the ringed birds, c. 15,000 are subsequently reported each year, either dead or alive away from the original site of ringing, by members of the public or other ringers. For all such records, the date and place of recovery are reported and, for many of the birds found dead, a putative cause of death (COD) is also given based on observations recorded by the finder. To quantify large-scale spatial and temporal variation in greenfinch mortality, the species most frequently affected by trichomonosis, records were examined for a total

[2] The British and Irish Ringing Scheme is funded through a partnership between the BTO, the Joint Nature Conservation Committee (on behalf of Natural England, Scottish Natural Heritage, the Countryside Council for Wales and the Council for Nature Conservation and the Countryside in Northern Ireland), BirdWatch Ireland and the ringers themselves.

of 2,206 recoveries of marked greenfinches found dead between 1st June and 30th November, the period of peak trichomonosis-related mortality, for the years 1996 to 2007 (inclusive). Longitudinal spread from the main area of the initial trichomonosis epidemic (central England) was the focus of the study, therefore, records of birds found dead north of 55°N (approximating the latitude of Newcastle-upon-Tyne) were excluded from the analyses.

Ring return records were classified according to the standardised reporting codes of the Euring code exchange scheme (Speek et al., 2001) based on the information submitted by the recorder. Post mortem examinations (PMEs) were not performed on the birds that form the ring return records: this dataset was independent to the disease surveillance undertaken by the GBH*i*. For the temporal analysis codes were aggregated into 5 suspected COD categories: 'predation', 'disease', 'natural causes' (mostly birds that looked emaciated with no obvious trauma, and so presumed starved but may be in poor body condition as a result of disease), 'collision' (mostly with road traffic or windows) and 'not established'. To compare between categories, the number of reports each year were standardised by the mean number of annual reports for each category in the period 1996-2005 (inclusive). For the spatial analysis, all reports of diseased greenfinches (n=69) were considered, those reported as dying due to 'natural causes' (n=17) and those for which the COD was 'not established' (i.e. there was no obvious sign of trauma or other reason for mortality) (n=853).

Surveillance for garden bird disease in Fennoscandia

Disease investigation of unusual mortality events in free-ranging wildlife in Fennoscandia is performed by governmental organisations. A comprehensive general surveillance programme covering the entire region has been in place for many decades. In all countries, reports of wild bird morbidity and mortality were received from members of the public and licensed bird ringers on an *ad hoc* basis. In Norway, carcasses are also submitted by inspectors belonging to the Norwegian Food Safety Authority. In Finland, disease events are also reported by those participating in the winter bird census, organised by the Finnish Museum of Natural History, which

monitors over 200 winter feeding places for birds (Väisänen et al., 1991). PMEs were performed on finch carcasses reported from multiple disease incidents in each country. Trichomonosis was diagnosed on the basis of characteristic lesions of necrotic ingluvitis along with the presence of trichomonad protozoa in wet preparations, or in trichomonad culture from lesions (Neimanis et al., 2010).

Molecular comparison of isolates from Great Britain and Fennoscandian finches

DNA was extracted from archived frozen necrotic ingluvitis lesions of Fennoscandian finches, or from positive parasite culture, using various extraction techniques including the one step 'miniprep' method for isolation of plasmid DNA (Chowdhury 1991) with either proteinase K digest and phenol/ chloroform solvent or the QIA amp DNA mini kit (Qiagen). Amplification and sequencing of the ITS1/ 5.8S rRNA/ ITS2 region and small subunit (SSU) rRNA gene was performed using the primers and cycling conditions described in Chapter 6 on DNA extracts from multiple Fennoscandian finches found dead in 2008 (Table 9.1). Sequence data from the Fennoscandian parasite isolates was compared with those from British greenfinches found dead in 2005-2006 (Chapter 6) to confirm the species of the parasite and determine whether the sequence data matched those from British finches.

Assessment of bird migration as a potential vector for parasite spread

Recoveries of birds ringed in Great Britain and found on mainland Europe were examined from 1980 to 2008 for greenfinch, chaffinch, wood pigeon (*Columba palumbus*) and collared dove (*Streptopelia decaocto*), these being the species in which trichomonosis was most frequently confirmed in Great Britain, to determine the extent of migration of each species between Great Britain and Fennoscandia. Available data for species less frequently affected by trichomonosis, particularly the goldfinch (*Carduelis carduelis*) and house sparrow (*Passer domesticus*), were also reviewed.

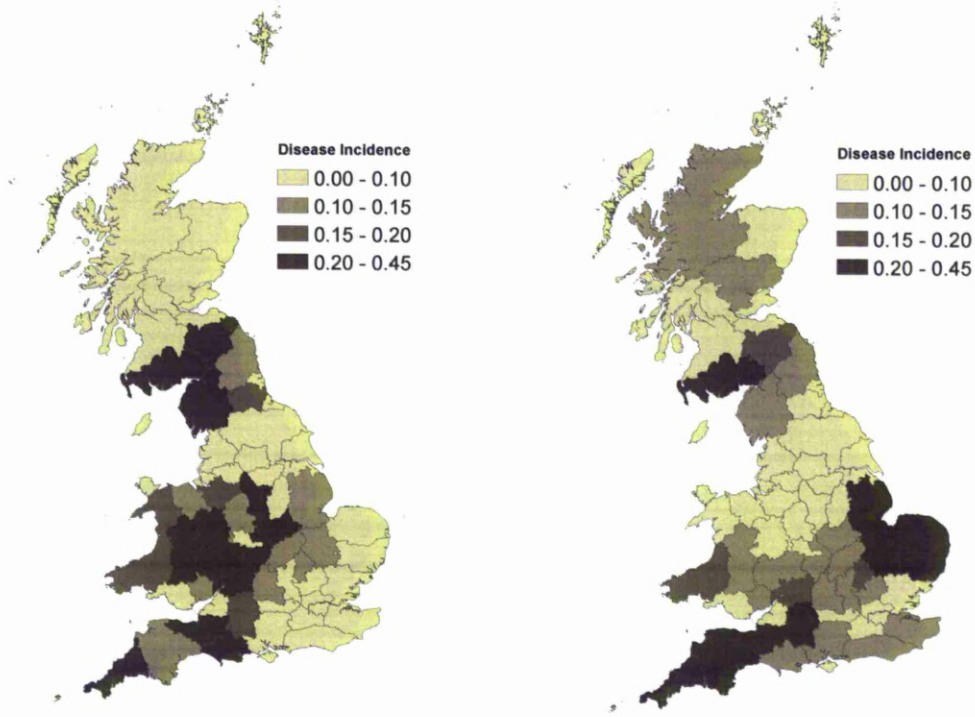
9.3 RESULTS

Pattern of greenfinch mortality

In Great Britain, a total of 1,054 trichomonosis incidents were recorded between 1st April 2006 and 30th September 2006 and 1,505 trichomonosis incidents were recorded between 1st April 2007 and 30th September 2007 through the opportunistic scheme according to pre-defined criteria. The seasonal peak for both years was in the autumn, however, the spatial distribution of the outbreak varied between years (Figure 9.1). Areas of epidemic finch mortality due to trichomonosis occurred in the central and western counties of England in 2006 and relatively few incidents were observed in the eastern region (Figure 9.1a). In 2007, trichomonosis spread to affect predominantly greenfinches in East Anglia and south-east England where high numbers of disease incidents were reported (Figure 9.1b).

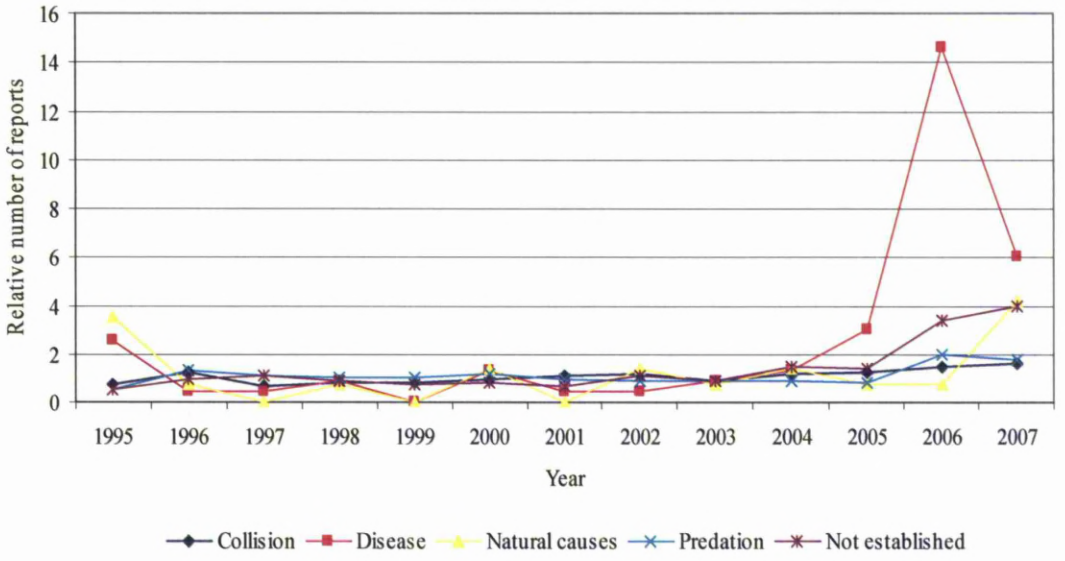
Figure 9.1: Incidence of suspected finch trichomonosis incidents per thousand households (according to the 2001 UK National Census (ONR 2005) during the periods (a) 1st April and 30th September 2006 and (b) 1st April and 30th September 2007.

(a) (b)



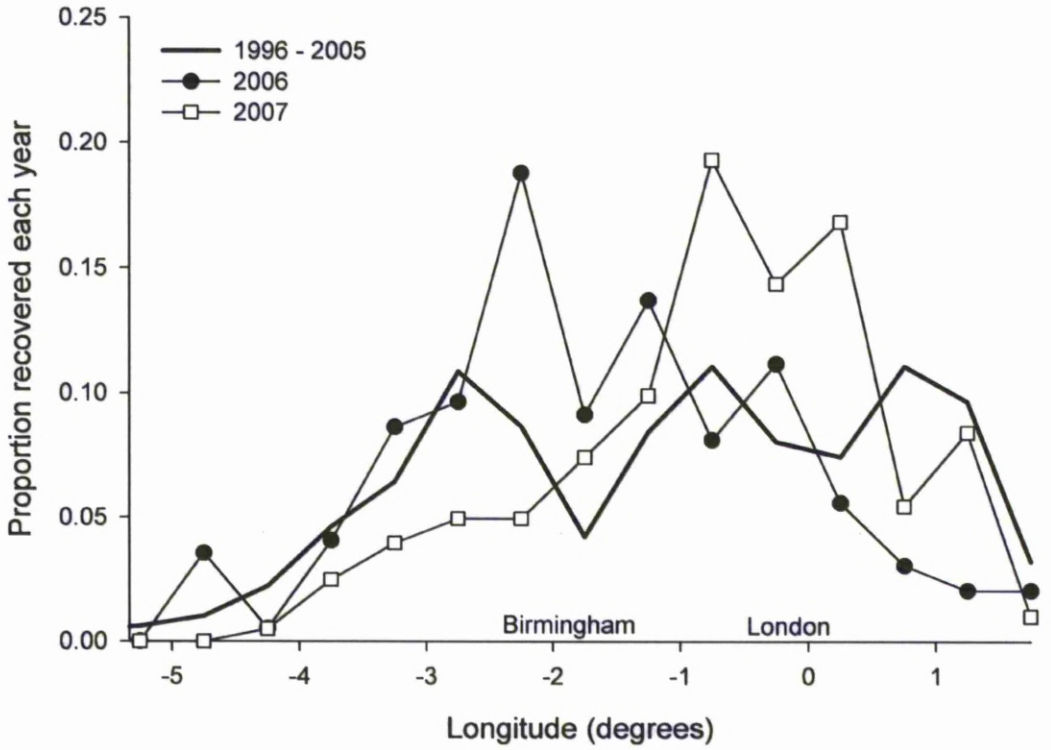
A significant increase in the number of ringed greenfinches found dead occurred with 336 in 2006 and 348 in 2007, as compared with a mean of 130 (range 107-157) in the previous 10 years (1996 – 2005). The number of individuals reported by the finder as having died as a result of disease was an order of magnitude greater in 2006-2007 combined (7% of all recoveries) than in the previous 10 years (1.6% of all recoveries, Figure 9.2). A small increase in the number of birds reported to have died of 'natural causes' or where the COD was 'not established' was seen in 2006-2007 (combined).

Figure 9.2: Number of ringed greenfinches reported in each year ascribed to particular COD categories: 'collision' (n=522), 'disease' (n=69), 'natural causes' (n=17), 'predation' (n=525), and 'not established' (n=853). Number of mortality reports expressed relative to the mean number reported annually in 1996-2005.



In the 10 years prior to the initial trichomonosis epidemic in 2006 mortality reports of ringed greenfinches were more or less evenly distributed across England with respect to longitude (Figure 9.3). In 2006, 60% of recoveries came from a region spanning 3.5°W (mid-Wales) to 1°W (Portsmouth and Nottingham), whereas in 2007, 66% of recoveries came from a region spanning 1.5°W (Southampton and Sheffield) to 1°E (east London) (Figure 9.3). The median longitude of recoveries shifted significantly eastwards from 1.75°W in 2006 to 0.75°W in 2007 (Wilcoxon rank-sum test $z=6.37$, $P<0.0001$).

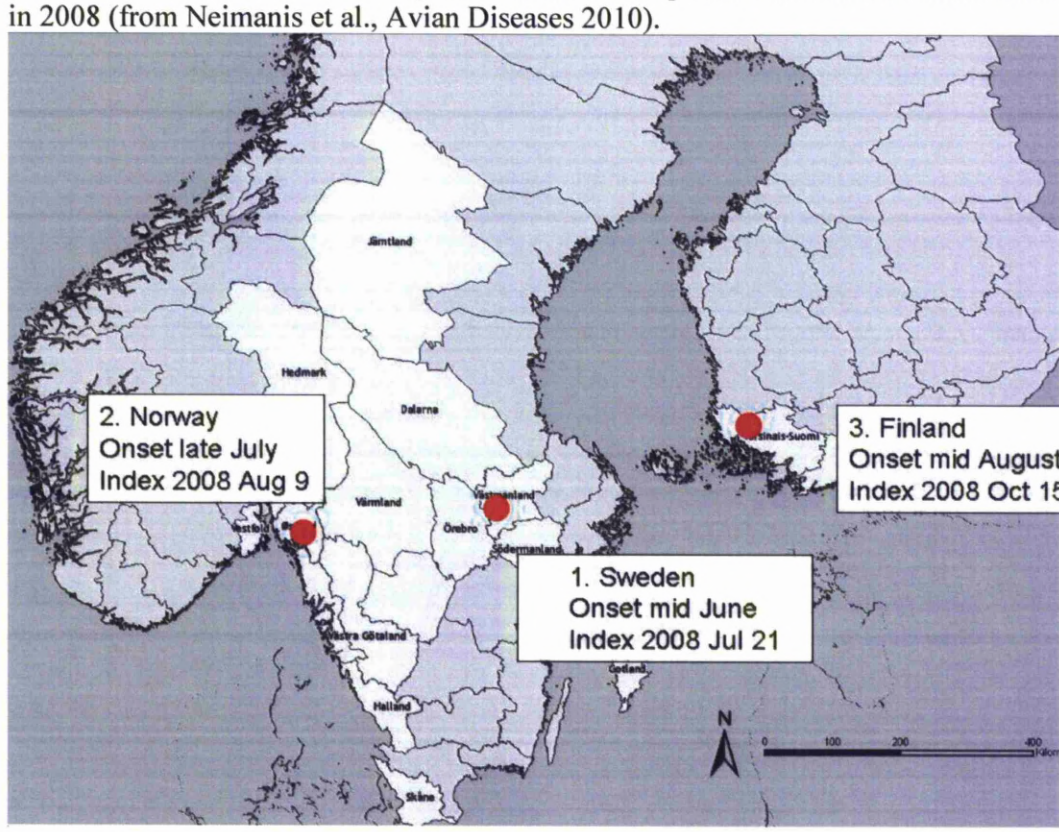
Figure 9.3: Percentage of dead greenfinches reported as ring recoveries by longitude during 1996-2005 (heavy line), 2006 (thin line with circles) showing peak of mortality in western England in 2006/07 and 2007 (thin line with squares) showing peak of mortality in eastern England in 2007/08.



Trichomonosis was first confirmed in a total of 20 finch mortality incidents in Finland, Norway and Sweden in 2008 and a further 20 finch mortality incidents were suspected to be caused by trichomonosis on the basis of clinical history, species affected and clinical signs (Neimanis et al., 2010). Greenfinches, and less commonly chaffinches, were the species mostly reported sick or dead in these incidents. In total 58 birds with trichomonosis were examined across Fennoscandia in 2008 of which 48 were greenfinches and 8 were chaffinches. As in Great Britain, finch trichomonosis incidents in Fennoscandia were almost always associated with bird feeding stations within private gardens.

The temporal pattern of the disease emergence in southern Fennoscandia is shown in Figure 9.4. Briefly, finch mortality incidents were first reported in Sweden in the municipality of Surahammar (59° 43' 43.79" N, 16° 06' 39.52" E) from mid June 2008, with the index case confirmed in a greenfinch found dead on 21st July 2008. The peak of mortality reports in Sweden occurred in August. In Norway, finch disease incidents were first reported in the eastern municipality of Hvaler (59°05' N, 10° 55' E), Østfold county from late July, with the index case confirmed in a greenfinch found dead on 9th August 2008. The peak of mortality reports in Norway occurred in August. In Finland, finch mortality was first reported in Kaarina (60°24' N, 22° 22' E) from mid-August with the index case confirmed in a greenfinch found dead on 15th October 2008. The peak of mortality reports in Finland occurred in October.

Figure 9.4: Emergence of finch trichomonosis in Fennoscandian finches.
Red dots mark the location of the index case in Sweden, Norway and Finland. Counties and provinces marked in white represent areas with reported outbreaks of finch mortality in 2008 (from Neimanis et al., Avian Diseases 2010).



Identification of disease organism in Fennoscandian finches

PCR amplification and sequencing of the ITS1/5.8S rRNA/ITS2 region from 11 cases (7 locations) (Table 9.1) identified a consensus sequence of 214 nucleotides for all PCR products identical to the sequence obtained from isolates from British greenfinches and chaffinches (Genbank accession no GQ150752 and GQ150753) (Chapter 6). NCBI BLAST identified 100% homology with 100% query coverage with 4 other reports of *T. gallinae* (EU215369, EU290649, EF208019, AY349182) confirming the species identification. Nested PCR amplification and sequencing of part of the SSU rRNA gene from 7 cases (7 locations) (Table 9.1) identified a consensus sequence of 149 nucleotides, also with 100% homology to those from British finches (Genbank accession no GQ214405) and with 4 other reports of *T. gallinae* (EU215372, EU215373, EU215374, EU215375).

Table 9.1: Details of the trichomonosis cases from which DNA was extracted for PCR amplification and sequencing.

Case Number	Species	Date found	Location	PCR and sequence of ITS1/ 5.8S rRNA/ ITS2 region	PCR and sequence of SSU rRNA gene
Finland 1	Greenfinch	10/10/2008	Turku, Finland	Yes	No
Finland 2	Greenfinch	05/11/2008	Turku, Finland	Yes	Yes
Norway 1	Greenfinch	22/08/2008	Hedmark, Norway	Yes	No
Norway 2	Greenfinch	04/09/2008	Akershus, Norway	Yes	Yes
Norway 3	Greenfinch	04/09/2008	Akershus, Norway	Yes	No
Norway 4	Greenfinch	25/09/2008	Oslo, Norway	Yes	Yes
Norway 5	Greenfinch	22/10/2008	Vestfold, Norway	Yes	Yes
Sweden 1	Greenfinch	03/08/2008	Vastervala, Sweden	Yes	No
Sweden 2	Greenfinch	03/08/2008	Vastervala, Sweden	No	Yes
Sweden 3	Chaffinch	14/08/2008	Surahammar, Sweden	No	Yes
Sweden 4	Greenfinch	06/10/2008	Nyhem, Sweden	Yes	No
Sweden 5	Greenfinch	22/10/2008	Bjursås, Sweden	Yes	Yes
Sweden 6	Greenfinch	28/10/2008	Nyköping, Sweden	Yes	No

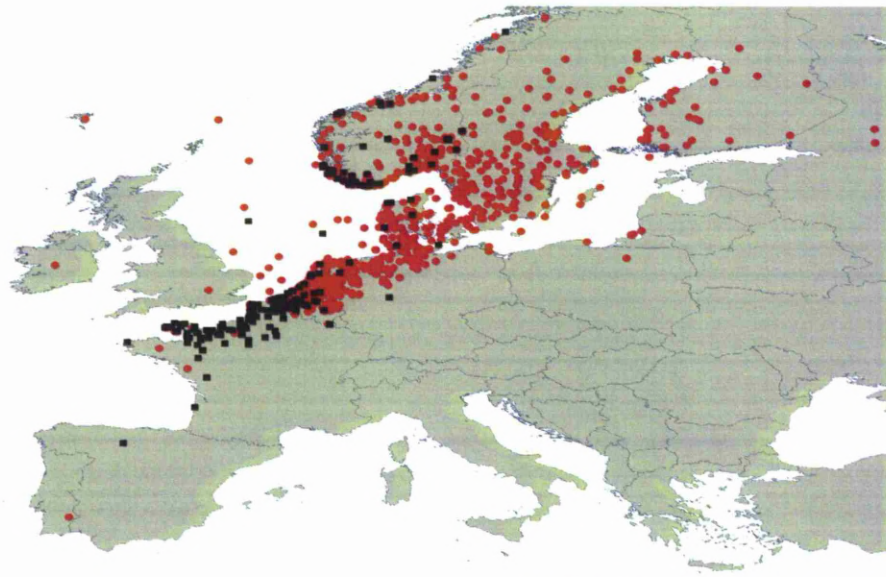
Avian migration routes

Many columbid and fringillid species are considered partial migrants. Greenfinches breeding in Norway (but not Sweden, which winter further south) that migrate spend the winter in Britain, the Netherlands and northern France (Figure 9.5a), whereas chaffinches from a much broader area of Fennoscandia overwinter (or migrate through) Britain (Figure 9.5a). The number of individuals that augment the resident British greenfinch and chaffinch populations is poorly known, however, migrants are thought to account for a small proportion of the overwintering greenfinch population (~1-2%) (Main 1999) but a considerable proportion of the chaffinch population (~30-50%) (Newton 1972; Lack 1986).

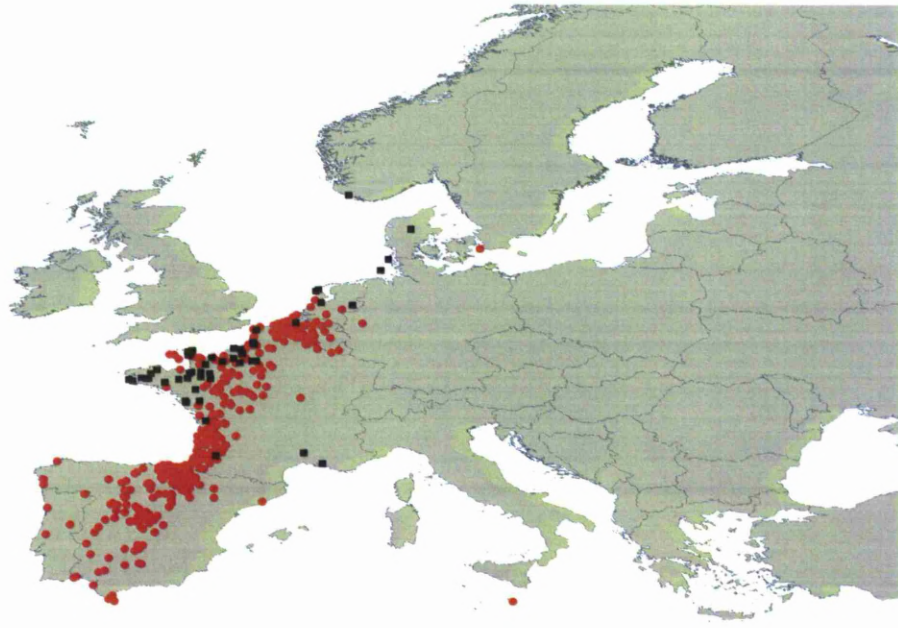
Of the other fringillids and columbids regularly occurring in gardens, goldfinches migrate south-west from Britain and the Netherlands, to spend the winter in western France and Iberia (Figure 9.5b). Although collared doves naturally colonised Britain in the 1950s from south-eastern Europe, they are generally sedentary (12 foreign recoveries from c. 34,000 birds ringed in Great Britain), with exchanges of birds only from the near continent. Similarly, although wood pigeons are quite mobile within Britain, known exchanges with the continent are few despite c. 45,000 birds having been ringed in Great Britain (Figure 9.5b). House sparrows show a remarkable degree of site fidelity and the 3 foreign recoveries (from c. 530,000 birds ringed) represent exceptional movements (Wernham et al., 2002).

Figure 9.5: Locations abroad of birds marked or found in Great Britain (1980 – 2008). (a) Greenfinch (black squares) and Chaffinch (red circles) and (b) Wood pigeon (black squares) and Goldfinch (red circles). Each point indicates the marking or finding of a bird that was found (or marked) in Great Britain; individuals appear only once on the map. Data from the British and Irish Ringing scheme, see Wernham et al. (2002) for details.

(a)



(b)



9.4 DISCUSSION

Identification of the epidemic

In Great Britain, trichomonosis, due to *T. gallinae* infection, first emerged in greenfinches and chaffinches in 2005, leading to epidemic mortality in 2006 and 2007, with an eastward shift in spatial distribution between these years. Ring return data from birds found dead supported the changing distribution of the epidemic observed from opportunistic reports of garden bird mortality incidents received from the public. A marked increase in the number of ring return birds reported as having died of disease occurred and a smaller increase in those reported to have died from 'natural causes' or for 'not established' reasons was also seen. However, the number of reports of birds involved in collisions or being predated did not increase appreciably. Thus a simple change in reporting behaviour is unlikely to be the cause of the changed number of submissions and birds found dead. Indeed, this increase is counter to the background trend of declining rates of ring recovery in British birds (Robinson et al., 2009).

Outbreaks of greenfinch and chaffinch mortality in Fennoscandia were first identified at multiple sites in the summer of 2008 (Neimanis et al., 2010). Pathological investigations, including amplification and sequencing of the ITS1/5.8S/ITS2 region which has been used to establish the identity of *T. gallinae* (Felleisen 1997; Gaspar da Silva et al., 2007, Gerhold et al., 2008), confirmed the causative agent as *T. gallinae*: the first time this parasite had been found to infect, and kill, finches in Fennoscandia. The Fennoscandian parasite had 100% sequence homology with *T. gallinae* isolated from British finches and with other *T. gallinae* ITS1/5.8S rRNA/ITS2 sequences on Genbank. Likewise, amplification and sequencing of part of the SSU rRNA gene, used as a diagnostic test in this study, found the Fennoscandian parasite had 100% sequence homology to British finch isolates and with other *T. gallinae* SSU rRNA gene sequences on Genbank. Whilst the temporal and spatial pattern of trichomonosis mortality indicates disease spread from Britain to southern Fennoscandia, further molecular analyses, such as random amplified polymorphic DNA studies and sequence analysis of the Fe hydrogenase gene (Chapter 8), to compare the finch *T. gallinae* isolates from both

regions, are required to determine if the parasite strain affecting Fennoscandian finches in 2008 is the same as that found infecting finches in Great Britain.

Finch trichomonosis had not previously been recognised in Fennoscandia despite established, long-term schemes for the investigation of wildlife mortality, and therefore the condition is considered to be a newly emergent disease in these countries. There was no record of an increase in the number of columbiform trichomonosis disease incidents in this region either prior to, or concurrent with, the emergence of finch trichomonosis nor was there a history of unusual reports of trichomonosis in captive avian collections (poultry, aviary or zoological collections). Year round garden bird feeding seems to have become more popular during recent years in Fennoscandia and in other countries in continental Europe. The potential for disease spread at shared food and water sources for wildlife species has been highlighted (Kirkwood 1998; Villanúa et al., 2006). Public education programmes to inform on best practice for garden bird feeding and disease control are important in the face of trichomonosis as an EID threat to European bird populations.

Role of bird migration in disease spread

Examples of disease spread through migratory movements of wild birds have been documented previously. Modelling data indicate that bird migration, in addition to mosquito vector spread, is likely to have contributed to the rapid, long-distance spread of West Nile virus from New York State in 1999 (Rappole et al., 2000; Peterson et al., 2003). British breeding populations of columbid and fringillid species (except goldfinch) migrate relatively short distances, typically only moving within the British Isles (Wernham et al., 2002). In 2006, the areas of highest mortality reports due to trichomonosis in finches were the central and western British counties. Within Britain, the greenfinch population is semi-migratory with partial movement towards the south-west in autumn and an eastward return the following spring (Main 1996). Whilst a marked seasonal peak occurred in the 2006 finch trichomonosis epidemic during the late summer and early autumn months, PMEs showed that disease incidents continued to occur throughout the winter months (GBH*i*, *unpublished data*) confirming that the

parasite persisted in British finch populations. Consequently, the shift in eastward distribution of the British finch trichomonosis epidemic from 2006 to 2007 may be explained through the seasonal movements of the greenfinch population back to this region in spring 2007 in advance of the breeding season and epidemic peak.

Although greenfinches were the species most frequently affected with trichomonosis, relatively few birds migrate from Fennoscandia (primarily Norway) to Britain, with many spending subsequent winters elsewhere, notably the Low countries (Figure 9.5a). In contrast, chaffinches migrate in relatively large numbers from breeding grounds in Fennoscandia to Britain. Ring return data also show that interchange of finches between Britain and Fennoscandia occurs most commonly with birds in the eastern counties of England, to which trichomonosis spread in 2007. Consequently both the temporal and spatial trends in spread of the epidemic support migratory finches as the principal disease vector and the chaffinch, which was the second most frequently diagnosed species, and which might also have suffered a high incidence of sub-clinical infection, presents the most plausible candidate as the primary vector of disease spread.

It is interesting to note that, whilst winter migrant chaffinches arrive in Fennoscandia for breeding in April, trichomonosis-associated mortality was not reported in the region until mid June 2008. This apparent lag phase between hypothesised parasite incursion and first reported mortality might indicate that the early phase of the epidemic was missed and that infection required a period to 'amplify' within local finch populations until notable mortality occurred: alternatively the delay in diagnosis might simply relate to chance with the opportunistic surveillance methods in place.

There is no evidence to support alternative explanations for the spread of finch trichomonosis from Great Britain to Fennoscandia. The protozoal parasite is highly labile and killed by desiccation (Erwin et al., 2000) and is therefore not amenable to long-distance mechanical transfer, as with some viruses (e.g. Nemeth et al., 2009; Yamamoto et al., 2009). There have been no reported trichomonosis cases in novel host species within captive environments (i.e. poultry or zoological collections) in Great

Britain since the emergence of this EID; consequently inadvertent parasite translocation through anthropogenically-mediated animal movement is also unlikely.

Strain variation of *Trichomonas gallinae* is well known in columbiform species where infection may be asymptomatic or subclinical, in addition to acting as a cause of morbidity and mortality (Bondurant et al., 1994). However, current understanding of the host reservoir for finch trichomonosis is limited and the extent to which the parasite may be spread by clinically healthy finches or columbiform species is unknown. In particular, the extent to which greenfinches or chaffinches with subclinical or mild clinical infection are capable of long-distance movement, resulting in spread of the parasite through migration, requires further investigation similar to that which has been undertaken with waterfowl species and H5N1 HPAI infection (Brown et al., 2008; Hars et al., 2008).

The situation in Fennoscandia in 2008, with sporadic small numbers of finch trichomonosis disease outbreaks, is similar to the situation seen in Great Britain in 2005 when the disease was first detected in finches there. Given the large greenfinch and chaffinch populations in Fennoscandia, the potential exists for epidemic mortality to occur in future years. Ring return data show the migration route of chaffinches from Scandinavia to Britain in the autumn involves birds flying through Denmark, Germany, the Netherlands and Belgium, before crossing the English Channel (Wernham et al., 2002); consequently surveillance in coastal regions of these countries should be vigilant for evidence of the disease. The return journey in spring typically is faster and is thought likely to be routed directly across the North Sea, which might explain why trichomonosis on the continent has first emerged in Fennoscandia rather than in, for example, the Low countries. The chaffinch and greenfinch are widespread in Europe and it is likely that the disease will now spread to these populations in other countries. As a wide range of bird species are susceptible to trichomonosis, disease surveillance should be performed for chaffinches, greenfinches, and other sympatric wild bird species likely to have contact with these bird populations across Europe, for evidence of this EID.

CHAPTER 10: EXPOSURE OF GARDEN BIRDS TO MYCOTOXINS IN GREAT BRITAIN

10.1 INTRODUCTION

Aflatoxins (AFs) are secondary metabolites of *Aspergillus* spp. which can exert acute toxic effects, principally targeting the liver, and chronic toxic effects, including impaired weight gain, reproductive and immune function (Pitt et al., 1997; Pier 1992). AFs frequently contaminate food products provisioned for wild birds in Great Britain, including peanuts, cereals and seeds (Gourama et al., 1995; Creekmore 1999; Chapter 11).

Experimental dietary challenge studies reveal marked and unpredictable variation in the susceptibility of bird species to aflatoxicosis (Ruff et al., 1990; Huff et al., 1992; Ruff et al., 1992). For example, a study by Henke et al. (2004) on the northern cardinal (*Cardinalis cardinalis*), a passerine species that frequents feeding stations in the U.S.A., revealed acute and chronic aflatoxicosis at dietary AF concentrations an order of magnitude lower than those observed to have adverse effects for sympatric game bird species. There is currently no information available on the susceptibility of native species in Great Britain to AF exposure.

Acute aflatoxicosis has caused mass mortality of waterfowl species in the U.S.A. (Robinson et al., 1982; Cornish et al., 1999); however there have been no confirmed cases of acute aflatoxicosis in wild birds in Great Britain. Recently, concern has been expressed about chronic low level exposure of AFs to wild bird species in the U.S.A., either through supplementary, agricultural or wild seed sources (Schweitzer et al., 2001). The possibility that chronic AF exposure might impair immune function and predispose wild birds to infectious disease in Great Britain requires investigation.

Recently, the UK Food Standards Agency (FSA) consulted on a proposal to decrease the maximum permissible level (MPL) of aflatoxin B1 (AFB_1) in wild bird food from the current 20 μg/kg, which relates to livestock foodstuffs (FSA 2003). The

consultation concluded that there was no available information on the significance of wild bird exposure to AFs in Great Britain and therefore no data to support a change in the current MPL.

To investigate if AF exposure is a risk for garden birds in Great Britain, AF analyses were conducted on liver samples collected from a subset of garden birds which had been submitted to the Institute of Zoology, London, for post mortem examination (PME) between 1999 and 2003. Greenfinches (*Carduelis chloris*) and house sparrows (*Passer domesticus*) were chosen for analysis as these 2 species are among the most common granivorous garden birds in Great Britain, are commonly submitted for PME and represent species that have increasing (greenfinch) and declining (house sparrow) populations (Baille et al., 2005). The liver was selected for AF testing because this organ retains the highest levels of AFs following ingestion (Madden et al., 1995) and is the major target organ in terms of the toxins' pathological effects (Oliveira et al., 2002).

10.1 MATERIALS AND METHODS

Opportunistic reports of garden bird mortality were solicited from members of the public through an organisational network comprising the British Trust for Ornithology, the Royal Society for the Protection of Birds, the Royal Society for the Prevention of Cruelty to Animals, the Universities Federation of Animal Welfare and the Zoological Society of London. When available, carcasses were submitted to the Institute of Zoology, where they were examined following a standardised PME protocol. Details of the date found, geographical origin and circumstances of death were recorded. Each bird was assigned a unique post mortem reference code. During each examination, the species, age, sex, total body weight and body condition were recorded. Systematic internal and external examination of body systems was performed and gross lesions described. Where indicated, and where the state of carcass decomposition permitted, samples were taken for parasitological, microbiological and toxicological investigations (Chapter 2 & 4). For the last of these, an archive of liver samples was kept in frozen storage at –20 ^{o}C. No cases were considered to be fresh enough for meaningful histopathological examination.

Liver, small intestine and any lesions observed were routinely sampled aseptically and examined for the presence of pathogenic bacteria using a standard protocol (Chapter 2&4). *Salmonella* spp. isolates were submitted to the Salmonella Reference Unit, Health Protection Agency, for complete biotyping according to standardised international protocols (Anderson et al., 1977) (Chapter 2 & 4).

Liver samples from 13 greenfinches (13 mortality incidents) and from 22 house sparrows (16 mortality incidents) found dead between 1999 and 2003 (Table 10.1) were analysed by Susan Macdonald and Sharron Anderson at the Food and Environment Research Agency (Fera, formerly the Central Science Laboratory) for the major AF residues (AFB_1, AFB_2, AFG_1, AFG_2) using a fully validated United Kingdom Accreditation Service (UKAS) high pressure liquid chromatography (HPLC) method based on published methods for AFs (Sharman et al., 1991; Trucksess et al., 1979; AOAC 1995).

To summarise, samples were blended at high speed with chloroform and 0.1M phosphoric acid solution. After centrifugation, the chloroform extract (or an aliquot of it) was evaporated to dryness and the residue redissolved in methanol and phosphate buffered saline. After de-fatting with hexane an aliquot of the aqueous extract was cleaned up by an Easi-Extract aflatoxin immunoaffinity column (R-Biopharm Rhone Ltd., Glasgow). The cleaned-up extract was analysed by reversed phase HPLC with fluorescence detection and post-column derivatisation with pyridinium bromide perbromide for AFB_1 and AFG_1. The immuno-affinity column clean-up and HPLC injection were carried out automatically by a Gilson ASPEC (Gilson Inc, U.S.A.)

Quantification was by external 4-point calibration using calibration standards prepared for each batch of samples. Peaks were identified by matching retention times to the nearest calibration standard. The specificity of the clean-up, derivatisation method and specific excitation and emission wavelengths used were considered sufficient to ensure accurate peak identification. Duplicate spiked samples were analysed in each batch, recovery values were in the range 70-93% (acceptable range 70-110%). All results met quality assurance parameters (e.g. peak

asymmetry, retention time drift, resolution) established for UKAS accredited AF analyses. Analyses were performed in 2 batches with variable residue detection limits according to sample volume and equipment calibration.

The AF analyses results were explored by cause of death (COD) and by season of death: winter (Dec-Feb), spring (Mar-May), summer (Jun-Aug) or autumn (Sept-Nov) (Table 10.1). Geographical location data was explored using ArcView 3.0 software (Environmental Systems Research Institute Geographical Information Systems and Mapping Software, Redlands, U.S.A.).

10.3 RESULTS

The results of the PMEs are summarised in Table 10.1. COD was categorised, in decreasing order of occurrence, as: 'salmonellosis', 'predation', 'other trauma' and 'not established'. All birds in this study that died of infectious disease did so of salmonellosis (9 greenfinches, 9 house sparrows). No other infectious COD was found.

No	Species	Season and year of death	Cause of death	AF residues measured† µg/kg (ppb)				
				AFB1	**AFB2**	**AFG1**	**AFG2**	**Total AF**
1	greenfinch	Winter 1999/ 2000	salmonellosis	0.1	ND	ND	ND	0.1
2	house sparrow	Summer 2000	non-infectious	ND	ND	ND	ND	ND
3	house sparrow	Autumn 2000	salmonellosis	0.8	0.1	ND	ND	0.9
4	house sparrow	Autumn 2000	salmonellosis	0.4	ND	ND	ND	0.4
5	greenfinch	Winter 2000/ 2001	salmonellosis	ND	ND	ND	ND	ND
6	greenfinch	Winter 2000/ 2001	salmonellosis	ND	ND	ND	ND	ND
7	greenfinch	Spring 2001	non-infectious	ND	ND	ND	ND	ND
8	greenfinch	Winter 2001/ 2002	salmonellosis	0.3	ND	ND	ND	0.3
9	greenfinch	Winter 2001/ 2002	salmonellosis	ND	ND	ND	ND	ND
10	greenfinch	Winter 2001/ 2002	not established	ND	ND	5.8	2.2	8.0
11	greenfinch	Winter 2001/ 2002	salmonellosis	0.1	ND	ND	ND	0.1
12	greenfinch	Winter 2001/ 2002	salmonellosis	0.2	ND	ND	ND	0.2
13	greenfinch	Winter 2001/ 2002	salmonellosis	0.1	ND	ND	ND	0.1
14	greenfinch	Winter 2001/ 2002	salmonellosis	0.7	0.1	ND	ND	0.8
15	house sparrow	Winter 2001/ 2002	salmonellosis	ND	ND	ND	ND	ND
16	house sparrow	Winter 2001/ 2002	salmonellosis	ND	ND	ND	ND	ND
17	house sparrow	Winter 2001/ 2002	salmonellosis	ND	ND	ND	ND	ND
18	house sparrow	Winter 2001/ 2002	salmonellosis	ND	ND	ND	ND	ND
19	house sparrow	Winter 2001/ 2002	salmonellosis	ND	ND	ND	ND	ND
20	house sparrow	Winter 2001/ 2002	salmonellosis	0.4	ND	ND	ND	0.4
21	house sparrow	Winter 2001/ 2002	salmonellosis	0.4	ND	ND	ND	0.4
22	greenfinch	Spring 2002	non-infectious	ND	ND	ND	ND	ND
23	house sparrow	Spring 2002	non-infectious	ND	ND	ND	ND	ND
24	house sparrow	Spring 2002	non-infectious	ND	ND	ND	ND	ND
25	house sparrow	Spring 2002	non-infectious	ND	ND	ND	ND	ND
26	house sparrow	Spring 2002	non-infectious	ND	ND	ND	ND	ND
27	greenfinch	Summer 2002	non-infectious	ND	ND	ND	ND	ND
28	house sparrow	Summer 2002	non-infectious	ND	ND	ND	ND	ND
29	house sparrow	Summer 2002	non-infectious	ND	ND	ND	ND	ND
30	house sparrow	Summer 2002	non-infectious	ND	ND	ND	ND	ND
31	house sparrow	Summer 2002	non-infectious	ND	ND	ND	ND	ND
32	house sparrow	Summer 2002	non-infectious	ND	ND	ND	ND	ND
33	house sparrow	Autumn 2002	non-infectious	ND	ND	ND	ND	ND
34	house sparrow	Summer 2003	non-infectious	ND	ND	ND	ND	ND
35	house sparrow	Summer 2003	non-infectious	ND	ND	ND	ND	ND

Table 10.1: Results of AF screening of liver samples from garden birds.† ND = not detectable

The causes of death reflect those most commonly found for greenfinches and house sparrows in a review of 11 years (1993–2003) of garden bird PMEs at the Institute of Zoology (Chapter 2). Salmonellosis was diagnosed on the basis of gross PME findings consistent with salmonellosis, coupled with the isolation of *Salmonella* Typhimurium from lesions (Pennycott et al., 1998a). None of the birds that died from predation or 'other trauma' (3 greenfinches, 13 house sparrows) had any evidence of infectious or other concurrent disease processes on pathological examination, including an absence of significant findings obtained on microbiological and parasitological examinations. These cases, therefore, have been considered together as a non-infectious COD group. The COD of 1 greenfinch was 'not established'.

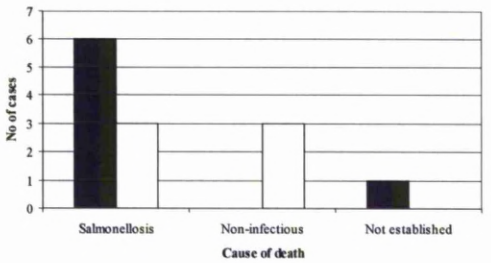
AF residues above detectable thresholds were identified in 4 of 22 house sparrows and in 7 of 13 greenfinches examined (Table 10.1). AFB_1 was the principal residue found in all but 1 of these 11 positive cases, with values ranging between 0.1 - 0.8 μg/kg. AFB_2 was detected only in the 2 birds with the highest levels of AFB_1. No gross evidence of hepatic disease consistent with either acute or chronic aflatoxicosis, such as icterus, hepatic pallor or discolouration (Robinson et al., 1982; Pier 1992) was observed in any of the birds examined.

AFG_1 and AFG_2 residues were found in 1 greenfinch only, with a total hepatic AF level of 8.0 μg/kg. No detectable levels of AFB_1 or AFB_2 were found in this bird. Unfortunately, the COD could not be established for this bird due to the extent of carcass decomposition; gross and microbiological examinations were negative for evidence of infectious disease, such as salmonellosis.

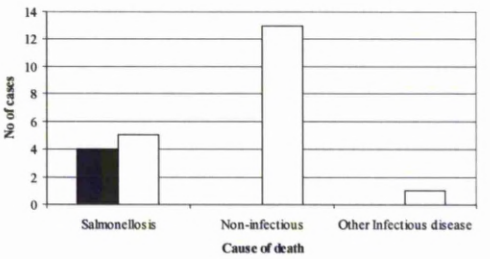
For both species examined, AFB_1 and AFB_2 residues were detected only in birds that had died as a result of infectious disease (salmonellosis). All tissues examined from birds in the 'non-infectious' group (i.e. birds that had died as a result of predation or 'other trauma') were negative for AF residues (Figure 10.1).

Figure 10.1: Number of (a) greenfinches (n=13) and (b) house sparrows (n=22) screened for AF residues by COD. Black columns represent birds positive for residues. White columns represent birds negative for residues.

(a) Greenfinches



(b) House sparrows

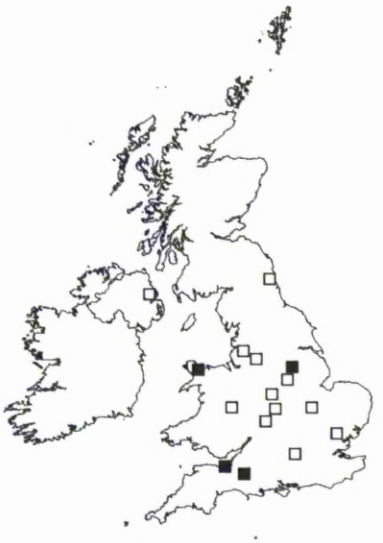


Only a small number of birds were examined in this study, but although there was no evidence of geographical (Figure 10.2) or temporal clustering of cases with detectable AF residues there was a strong seasonal influence.

Figure 10.2: Geographical distribution of (a) greenfinches (n=13 locations) and (b) house sparrows (n=16 locations) screened for AF residues. Each bold square represents the location of a bird that tested positive for residues. Each empty square represents the location of a bird (or birds) that tested negative for residues.

(a) Greenfinches

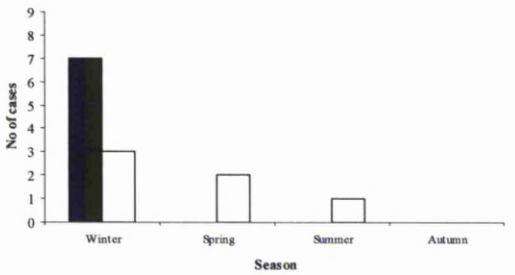
(b) House sparrows



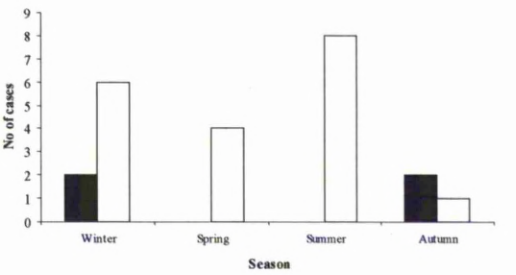
Detectable levels of AF were found only in birds that died between November and February (Figure 10.3). Detectable residues were found in birds that died in 1999 (1 of 1 case), 2000 (2 of 5 cases) and 2001 (8 of 15 cases), whilst no residues were detected in birds examined in 2002 (0 of 12 cases) or 2003 (0 of 2 cases).

Figure 10.3: Number of (a) greenfinches (n=13) and (b) house sparrows (n=22) screened for AF residues by season. Bold columns represent birds positive for residues. White columns represent birds negative for residues. Winter (Dec – Feb); spring (Mar – May); summer (June – Aug); autumn (Sept – Nov).

(a) Greenfinches



(b) House sparrows



10.4 DISCUSSION

Hepatic AF residues were identified in 4 of 22 house sparrows and in 7 of 13 greenfinches examined. This is the first evidence that wild birds are exposed to AFs in Great Britain.

The order of toxicity of the AFs is $AFB_1 > AFG_1 > AFB_2 > AFG_2$ (Gourama et al., 1995). AFB_1 is the most commonly occurring and the most toxic of the group of AFs (Creekmore 1999) and this was the toxin most frequently identified in the current study. AFB_2 was detected in the 2 birds with the highest levels of AFB_1. The only bird for which the COD could not be established had by far the highest levels of AFs in its liver. This bird, a greenfinch, had high levels of AFG_1 and AFG_2: this finding is unusual in the absence of other AF residues therefore the biological significance of this finding is unclear.

The absence of any gross evidence of toxicity and the inability to conduct meaningful histopathological examinations on the birds tested for AF exposure makes interpretation of the results difficult. There is little reported in the literature on hepatic AF levels in birds and the majority of these reports are based on experimental dosing of avian species (domestic and wild) with known dietary concentrations of AFs followed by monitoring of the physiological or pathological effects observed. Little information is available regarding the relationships between dietary AF levels and resultant tissue AF residues for bird species, particularly in combination with an assessment of the AFs' pathological significance.

In some studies, however, correlations have been made between dietary AF concentrations and the subsequent concentrations of tissue residues, although often the latter are at very low levels, in the order of less than several μg/kg (Gregory et al., 1983). Bintvihok et al. (2002) fed a variety of domestic poultry species with diets containing 3000 μg/kg AFB_1 for a 7-day period and showed that levels of AFB_1 were greater in liver than in muscle for all bird species tested. The ratio of AFB_1 toxin in the feed to the residual level in the liver varied between 383:1 for quail and ≥ 5769:1 for ducks and chickens: study birds were sacrificed either 24 or 72 hours after the last dietary AF exposure. Trucksess et al. (1983) fed a diet containing 8000 μg/kg AFB_1 to hens for 7 days and also detected greater concentrations of AFB_1 in the liver than other tissues (muscle, kidney), ranging from 0.11-0.83 μg/kg AFB_1 in the 9 birds within the study: this represents an average AF conversion factor from feed to liver of 17778:1 study birds were sacrificed on the last day of dietary AF exposure. Madden et al. (1995) fed 700 μg/kg AFB_1 to 14 day-old white leghorn chicks for 28 days and demonstrated that the highest levels of AFB_1 were found in the liver (1.29 μg/kg), followed by the crop (0.074 μg/kg) and muscle tissue (0.014 μg/kg) with an AF conversion factor from feed to liver of around 543:1: study birds were sacrificed on the last day of dietary AF exposure.

In another experiment to evaluate rates of AF residue clearance, Gregory et al. (1983) fed day-old turkey poults on a diet containing 500 μg/kg of AFB_1 for a period of 18 days, followed by a variable withdrawal period on an AF-free control diet. Results showed that tissue levels of AF residue were greater in liver than in muscle tissue, although all tissue residues were at a low level (range 0.01-1.19 μg/kg): study

birds were sacrificed at day 0, 1, 2 and 3 following the last day of dietary AF exposure. PME of the poults on this diet revealed slight liver hypertrophy, mild petechial haemorrhages and occasional tan discolouration of the liver:

These AF feeding experiments indicate that the metabolism of dietary AF extrapolates to a much smaller corresponding level in liver tissues with a conversion ratio that is highly variable according to species and other factors. Consequently, it is likely that the levels of hepatic AF residues found in British wild birds in the current study result from much higher levels of dietary exposure than the levels detected in the liver samples tested.

Dietary AFs are rapidly metabolised and excreted from the body, both in free and conjugated forms. For example, a study in turkey poults determined a half-life of 1.4 days for total AF residues in the liver (Gregory et al., 1983). Whilst the half-lives for hepatic AFs in the house sparrow and greenfinch are unknown, the rapid elimination in another avian species suggests that the birds in this study were exposed to contaminated dietary sources in the recent period prior to death. The exposure of garden birds in Great Britain to AFs, therefore, is likely to be more frequent and widespread than the results of this study indicate.

Chronic AF exposure has been shown to produce histopathological abnormalities such as mild biliary hyperplasia, periportal fibrosis and hepatocellular lipidosis (Pier 1992; Ortatatli et al., 2005). Appraisal of liver histopathology in tandem with AF screening would be a logical next stage in determining the significance of AF exposure in British wild birds.

It has been hypothesised that immunosuppression, with a resultant predisposition to infectious disease, might be one of the most important effects of AFs on wild turkeys (Quist et al., 2000). This might also be true for other free-ranging species exposed to sub-lethal doses of AFs. In the current study, AFB_1 residues were detected only in garden birds that had died as a consequence of infectious disease (salmonellosis) and it is tempting to speculate that AF-mediated immunosuppression might be a contributory factor to this finding. Devegowda et al. (2005) specifically cited increased susceptibility to salmonellosis as a consequence of subacute aflatoxicosis

in poultry. However, salmonellosis has a highly seasonal occurrence in garden birds in Great Britain, peaking during the winter months (Pennycott et al., 1998a; Chapter 2 & 4) and all AFB_1-positive birds in the current study died between November and February. The toxicological examination of birds that die of non-infectious causes in the winter months and of birds that die of infectious disease during the rest of the year is required in order to separate the confounding variables of infectious disease and season. Further research is required to determine the source of AFs for garden birds in Great Britain. It might be that exposure of garden birds to AFs is greater during the winter, perhaps through their increased reliance on supplementary feeding during this period. Further research also is required to investigate the pathological significance, if any, of AFs to wild birds in Great Britain.

CHAPTER 11: AFLATOXIN AND OCHRATOXIN A IN SUPPLEMENTARY FOODS USED FOR WILD BIRD CONSUMPTION IN GREAT BRITAIN

12.1 INTRODUCTION

Aflatoxins (AF) and ochratoxin A (OA) are secondary metabolites produced by filamentous fungi of the genera *Aspergillus* and *Penicillium* that have been shown to be produced in peanuts and other foodstuffs used in supplementary foods sold for garden bird consumption (Sweeney et al., 1998). AF and OA exert a range of adverse effects in birds, ranging from acute toxicosis to chronic subclinical impairment of growth, reproduction and immune function (Hamilton et al., 1982; Sweeney et al., 1998; Qureshi et al., 1998; Creekmore 1999; Quist et al., 2000).

Chapter 10 described the detection of AF residues in the liver of British house sparrow (*Passer domesticus*) and greenfinch (*Carduelis chloris*) examined post mortem from 1999-2003. This confirms that British garden birds are exposed to AFs, however, the pathological significance of this, if any, is uncertain. No research into OA residues in wild bird tissues appears to have been performed.

Currently, the source of AF exposure for garden birds is unknown. Possible sources include supplementary food contaminated with AF at the point of sale; contaminated after sale from production during storage; contaminated after sale from production whilst exposed to British climatic conditions at the feeding station itself, or a combination of the above. Alternatively, the source of the AF may be agricultural crops or wild food (grass seeds etc.).

Detectable levels of AF and OA have been found in surveys of wild bird food in the UK (Scudamore et al., 1997). Current UK legislation stipulates a maximum permitted level

(MPL) of 20 μg/kg AFB_1 in peanuts for livestock feed, including wild bird feed (Anon. 2004a; Anon. 2004b; Anon. 2004c; Anon. 2004d). There are no specific MPL's in place for AFB_1 in other wild bird foodstuffs although a Food Standards Agency (FSA) consultation emphasised a need for research on the significance of wild bird exposure to AF in Great Britain (FSA 2003). No MPL exists for OA, although European Union (EU) Commission Recommendation 2006/576/EC (Anon. 2006) provides a Guidance Limit of 100 μg/kg for poultry foodstuffs.

Research in poultry species has shown marked interspecific variation in the toxic effects of mycotoxins (e.g. Ruff et al., 1992) and no information is currently available on the susceptibility of British wild bird species. Industry-led consortia have applied the precautionary principle and agreed best practice standards, adopting nil detectable AF residues in the peanuts for sale (BSA 2009). Branded peanuts frequently provide labelling on the packaging to indicate that the contents have been screened for AF residues. However, peanuts for wild bird consumption are also sold from a variety of sources where clear labelling is not available, consequently, the source and quality control procedures followed are unknown.

This chapter explores the hypothesis that AF levels at the point of sale are lower in peanuts sold for wild birds in branded products that have been screened for AF compared to non-branded peanuts where detailed information on quality control procedures is not available.

Optimal conditions for AF production occur at high temperature and relative humidity; however, Thompson et al. (2000) demonstrated AF production under temperate climatic conditions that regularly occur in Great Britain. OA production also occurs in temperate conditions as found in the UK (Santin 2005). Historically, wild bird foods were provided during the winter months in British gardens, but over the past decade there has been a move to include summer feeding when higher ambient temperatures appropriate for AF production occur (Routh et al., 1995). Consequently, there is a potential risk for

production of both AF and OA within feed products at garden bird feeding stations and a need to determine whether this occurs in Great Britain, and to quantify the extent of this risk of exposure.

This chapter also explores the hypothesis that AF or OA production occurs in wild bird seed or peanuts in British climatic conditions at garden feeding stations and that production of these mycotoxins occurs in wild bird peanuts under storage conditions after the point of sale.

A pilot study screening food residues collected from hanging feeders, in use at feeding stations in south-eastern England, for mycotoxins was also performed. A confidential questionnaire on garden bird feeding methods was used to evaluate how closely the conditions simulate current practice.

12.2 MATERIALS AND METHODS

EXPERIMENT 1 - PILOT STUDY TO INVESTIGATE MYCOTOXIN PRESENCE AND PRODUCTION IN WILD BIRD FEED IN BRITAIN

Sampling protocol

Five kilograms of a non-branded wild bird feed mix (containing seeds and cereal grains) was purchased from a pet shop, and 5 kg of non-branded peanuts intended for wild bird consumption were purchased from a pet food street stall. In addition, 4.8 kg of a branded wild bird feed mix (containing sunflower seeds and hearts, maize, millet, chopped peanuts, pinhead oatmeal, and canary seed) and 5.6 kg of a proprietary brand of wild bird peanuts (labels state that the product was accredited to be 'nil detectable' AF) were purchased via mail order. Eighteen 21 cm long plastic 'Discovery' hanging seed feeders

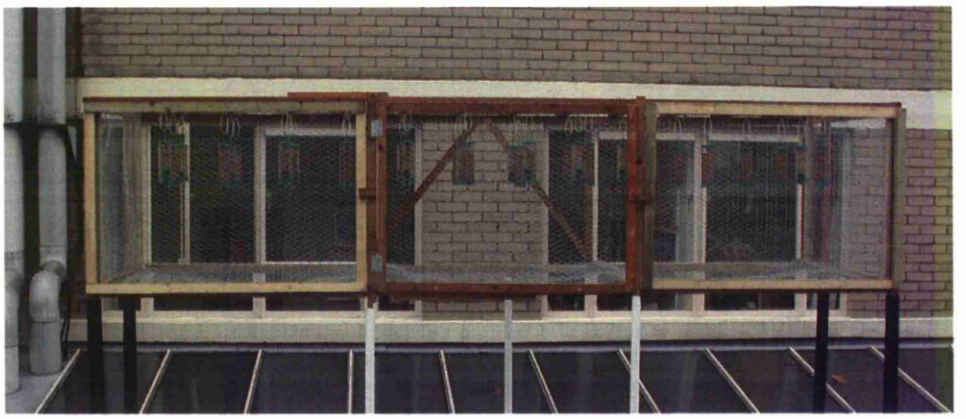
(CJ Wildlife, UK) and eighteen 21 cm long 'Discovery' hanging wire mesh peanut feeders (CJ Wildlife, UK) were obtained.

The non-branded wild bird feed mix was mixed thoroughly and six 50 g control samples were taken at random. These samples were placed in air- and watertight re-sealable freezer bags and frozen at -20 °C. Nine seed feeders were then filled at random from the remaining non-branded feed mix. Each feeder contained approximately 300 g of food. This procedure was repeated for each of the 3 other bird food products.

Exposure of samples to UK climatic conditions

The feeders were suspended within a mock feeding station constructed from plywood and wire mesh (c. 5 metres long by 1.5 metres wide by 1 metre high and raised 1 metre above ground level – Figure 11.1) which was situated on a roof at the Institute of Zoology (Regent's Park, London). Inside this structure, the contents of the feeders were exposed to climatic conditions such as wind and rain, but access by birds and mammals was prevented. A digital 'Memory Thermo-Hygrometer' (ATP Instrumentation Ltd., Leicestershire) was placed beneath the feeding station in a raised position and protected from rain. Daily maximum and minimum relative humidity (%) and temperature readings (°C) were recorded to monitor the climatic conditions to which the bird food was exposed. The study was carried out from 6th April to 29th June, 2005.

Figure 11.1: Feed samples in mock feeding station.



The feeders were hung in 3 rows, alternating peanut and seed feeders both along and between rows. The length of time for which the feeder in each position was exposed to UK climatic conditions (30, 60 or 90 days) was randomly assigned: accordingly a single feeder containing each food type was removed from each row every 30 days and the contents emptied into separate air- and watertight re-sealable freezer bags and stored at -20°C pending analysis for mycotoxins using high pressure liquid chromatography (HPLC) (see methods below). The findings from this pilot study were used to inform the protocol used for the main study where quantitative analyses were performed.

EXPERIMENT 2 - MAIN STUDY TO INVESTIGATE MYCOTOXIN PRESENCE AND PRODUCTION IN WILD BIRD FEED IN BRITAIN

Further to the results of the pilot study, a larger experiment was conducted on peanuts exposed to (1) 30 days of climatic conditions in spring, (2) 30 days of climatic conditions in summer, or (3) 90 days of storage.

Sampling protocol

An internet search was performed to identify companies supplying wild bird peanuts in Britain and information on their mycotoxin quality control procedures was collated. A

total of 40 peanut products (comprised only of peanuts) marketed for wild bird consumption were purchased in early 2007; 20 branded samples included information on the packaging which indicated that the contents had been screened for AFs and 20 were from non-branded sources where detailed information on quality control procedures was not available. Peanuts were purchased through mail order (n=14) and from a range of supermarkets (n=5), garden centres (n=2), pet shops and pet market stalls (n=19). Whole peanuts were used for 39 samples and peanut granules were used in 1 sample. Each sample was mixed thoroughly and four 250g aliquots were taken at random. Numbers (1 to 40) were assigned for each product and the remainder of the study was performed blind to the details of the commercial source.

Mycotoxin levels present at Point of Sale

Point of sale samples (Point of Sale treatment), representing baseline mycotoxin levels, were taken and individually stored in air- and water-tight plastic bags at -20 °C pending HPLC analysis.

Exposure to British climate conditions

A second batch ('Spring Exposure' treatment) was exposed to British climatic conditions for a period of 30 days in March/ April 2007 to emulate the end of the winter feeding season. Each sample was transferred to a 'Discovery' hanging wire mesh peanut feeder. The position of each sample in the mock feeding station was randomly assigned through blind ballot as in Experiment 1. Climatic variables were recorded as in Experiment 1. At the completion of the exposure period, samples were transferred to air- and water-tight plastic bags and stored at -20 °C pending HPLC analysis.

A third batch ('Summer Exposure' treatment) of each of the samples was exposed to British climatic conditions for a period of 30 days in June/ July 2007 to emulate summer feeding using the same protocol as per the Spring Exposure treatment. These samples

were stored at -20 °C for the period following purchase before this component of the trial began and again at the end of the treatment pending HPLC analysis.

Exposure to storage conditions

A fourth batch (Storage treatment) of samples was placed in storage in a cool and dry indoor room for a 90 day period (March-June 2007). Each sample was stored in a non-sealed plastic bag in a ventilated plastic crate. Samples were gently mixed on a weekly basis to simulate peanuts being removed from a packet of food. A digital Memory Thermo-Hygrometer was placed in the plastic crate and readings were taken on a weekly basis. At the completion of the exposure period, samples were transferred to air- and water-tight plastic bags and stored at -20 °C pending HPLC analysis.

Analysis

First, the degree of heterogeneity in the AF and OA levels found in the forty peanut samples, across each of the treatment categories, was assessed. Evidence for production of AF or OA under British climatic conditions or storage conditions was evaluated using the non-parametric Wilcoxon Signed-Rank Test for paired data comparing the results of the Point of Sale treatment with those for each of the other treatments in turn.

AF and OA levels in the branded screened peanuts were compared with those in the non-branded peanuts for each of the treatment categories using a qualitative approach to compare presence or absence of AF or OA (Pearson chi-square test or Fisher's Exact test) residues above the limit of detection. For the main study quantitative analyses were undertaken using the non-parametric Mann Whitney-U test to compare the absolute values of AF and OA. Samples which were negative for AF or OA at the limit of the detection were treated as zero for the purpose of these analyses.

Zoological Society of London (ZSL) members of staff were invited to participate in an anonymous survey of mycotoxin screening of the food residues collected from the base of the hanging feeders in use in their gardens. Respondents emptied the food contents from the bottom of their feeders when they were almost empty into a sterile universal tube and also scraped any residues into the container. Samples were submitted within 48 hours of collection and stored at -20 °C pending HPLC analysis. For each sample, the location, date of sampling, food type, and estimated duration of time that the food had been within the feeder (i.e. period since the feeder was last cleaned) was recorded. These samples were collected in August 2005.

High pressure liquid chromatography (HPLC)

Samples were transported frozen to the Food and Environment Research Agency (Fera, formerly the Central Science Laboratory) for HPLC analysis which was performed by Susan Macdonald and Sharron Anderson. Samples were stored at -20 °C and allowed to thaw overnight before preparation for analysis. A homogenised slurry was created from each sample by adding 200 ml of tap water per 250 g of food product (peanuts or mixed seed) and grinding the sample in a food processor for 20 minutes.

Analysis of mycotoxins were carried out using fully validated and accredited (to ISO 17025 by the United Kingdom Accreditation Scheme) methods based on those published for AF (Sharman et al., 1991) and OA (Sharman et al, 1992) or a combined AF/OA method (Chan et al., 2004) depending on the availability of clean-up columns: validation and quality control procedures have shown that single or combined methods produce equivalent results (Chan et al., 2004). To perform the extraction, 36 g of the slurried sample was homogenised at high speed with 84 ml of the extraction solution (containing acetonitrile / deionised water, 60:24, volume/volume). The extract was filtered and an aliquot was diluted with phosphate-buffered saline (PBS).

Fully automated immunoaffinity column clean-up and reverse phase HPLC with fluorescence detection was carried out on the resulting aqueous solutions as per the tissue analyses (Chapter 10). Samples were cleaned up by combined AF and OA immunoaffinity columns (AflaOchra) and the toxins analysed in one run (Chan et al., 2004) or the extracts were split and AFs and OA were cleaned-up and analysed separately, for AFs using the conditions used for the tissue analyses (Chapter 10) (Sharman et al., 1991) and for OA using the method of Sharman et al. (1992). Samples were run in batches of 20 on consecutive days with the same equipment and operator to minimize laboratory variability. In-house reference materials with known mycotoxin concentrations were used in each batch run to ensure that variation between batches remained within acceptable limits.

In each analytical run, a batch of 20 samples was tested, including a blank matrix sample and 2 spiked matrix samples. The concentrations (µg/kg) of mycotoxins detected were corrected according to the percentage of mycotoxin recovery achieved in each batch analysed, as determined from the results for the spiked samples. Recovery rates of 70 - 110% fall within the method performance criteria for AF and OA analysis at 1-10 µg/kg range set by the European Committee for Standardization (CEN 1999). Samples with no detectable mycotoxin residues above the level of detection were treated as zero for the purposes of statistical analysis.

Bird feeder residues were weighed and processed using the same protocol with fully automated immunoaffinity column clean-up and reverse phase HPLC with fluorescence detection. Appropriate dilutions of extraction solvents were used in proportion to sample size.

QUESTIONNAIRE ON GARDEN BIRD FEEDING PRACTICE

In order to determine how closely the conditions in this study simulated garden bird feeding practice in the UK, a confidential questionnaire was distributed via e-mail to employees of the ZSL (Regent's Park, London and Whipsnade Wild Animal Park, Bedfordshire) and of the Royal Society for the Protection of Birds (RSPB),

Bedfordshire, in August 2005. The target respondents were people who used hanging feeders to feed garden birds. Respondents were asked whether they purchased non-branded peanuts, branded peanuts, or a combination of both; whether they provided supplementary food during winter only (November – February), year-round or on a variable basis; and whether they usually topped up their feeders with food, emptied them before re-filling, or a combination of the two.

12.3 RESULTS

EXPERIMENT 1 - PILOT STUDY TO INVESTIGATE MYCOTOXIN PRESENCE AND PRODUCTION IN WILD BIRD FEED IN BRITAIN

During the study period the ambient temperature range was -1.5 - 36.3 °C and the relative humidity range was 25-99%. The mycotoxin results obtained for all of the feed mix and peanut samples (corrected according to mycotoxin recovery rates) are displayed in Tables 11.1 and 11.2. None of the samples were found to contain detectable levels of AFG_1 or AFG_2.

Table 11.1: Concentration of AFB_1 and B_2 and OA in each of the wild bird feed mix samples tested. Samples with detectable mycotoxin residues are highlighted in yellow.

Number of days exposed to climatic conditions	Non-Branded Feed Mix				Branded Feed Mix			
	Sample Number	Concentration (µg/kg)			Sample Number	Concentration (µg/kg)		
		AFB_1	AFB_2	OA		AFB_1	AFB_2	OA
0	Point of Sale 1	<0.2*	<0.2	<0.2	Point of Sale 1	<0.2	<0.2	<0.2
0	Pont of Sale 2	<0.2	<0.2	<0.2	Point of Sale 2	<0.2	<0.2	<0.2
0	Point of Sale 3	<0.2	<0.2	20.0	Point of Sale 3	<0.2	<0.2	<0.2
0	Point of Sale 4	<0.2	<0.2	<0.2	Point of Sale 4	<0.2	<0.2	<0.2
0	Point of Sale 5	<0.2	<0.2	<0.2	Point of Sale 5	<0.2	<0.2	<0.2
0	Point of Sale 6	<0.2	<0.2	<0.2	Point of Sale 6	<0.2	<0.2	<0.2
30	A7	<0.2	<0.2	4.7	A5	0.8	<0.2	<0.2
30	B6	<0.2	<0.2	<0.2	B4	0.4	<0.2	<0.2
30	C7	<0.2	<0.2	<0.2	C1	<0.2	<0.2	<0.2
60	A11	<0.2	<0.2	<0.2	A1	0.2	<0.2	<0.2
60	B10	0.4	<0.2	<0.2	B12	<0.2	<0.2	<0.2
60	C11	<0.2	<0.2	<0.2	C9	<0.2	<0.2	1.9
90	A3	<0.2	<0.2	<0.2	A9	<0.2	<0.2	<0.2
90	B2	<0.2	<0.2	<0.2	B8	<0.2	<0.2	<0.2
90	C3	<0.2	<0.2	<0.2	C5	<0.2	<0.2	<0.2

*Limit of quantification was 0.2 µg/kg for AFB_1, AFB_2, AFG_1, AFG_2, and OA

Table 11.2: Concentration of AFB_1 and B_2 and OA in each of the wild bird peanut samples tested. Samples with detectable mycotoxin residues are highlighted in yellow.

Number of days exposed to climatic conditions	Non-branded Peanuts				Branded Peanuts			
	Sample Number	Concentration (µg/kg)			Sample Number	Concentration (µg/kg)		
		AFB_1	AFB_2	OA		AFB_1	AFB_2	OA
0	Point of Sale 1	24.3	5.2	<1.5**	Point of Sale 1	<0.2	<0.2	<1.5
0	Point of Sale 2	0.5	<0.2*	<1.5	Point of Sale 2	<0.2	<0.2	<1.5
0	Point of Sale 3	0.5	<0.2	<1.5	Point of Sale 3	<0.2	<0.2	<1.5
0	Point of Sale 4	0.9	<0.2	<1.5	Point of Sale 4	<0.2	<0.2	<1.5
0	Point of Sale 5	0.6	<0.2	<1.5	Point of Sale 5	<0.2	<0.2	<1.5
0	Point of Sale 6	0.4	<0.2	<1.5	Point of Sale 6	0.5	0.2	<1.5
30	A8	0.4	0.2	<1.5	A6	1.5	0.2	<1.5
30	B9	0.7	0.2	<1.5	B3	<0.2	<0.2	<1.5
30	C4	0.7	<0.2	<1.5	C2	0.4	<0.2	<1.5
60	A4	0.3	<0.2	<1.5	A10	0.5	<0.2	<1.5
60	B1	341.0	49.4	<1.5	B11	<0.2	<0.2	<1.5
60	C8	0.5	0.5	<1.5	C10	<0.2	<0.2	<1.5
90	A12	<0.2	<0.2	<1.5	A2	<0.2	<0.2	<1.5
90	B5	1.4	<0.2	<1.5	B7	0.3	<0.2	<1.5
90	C12	<0.2	<0.2	<1.5	C6	0.7	<0.2	<1.5

*Limit of quantification was 0.2 µg/kg for AFB_1, AFB_2, AFG_1, AFG_2

**Corrected value for minimum quantifiable concentration (0.2 µg/kg) at average OA recovery rate of 13%

Recovery rates for OA in the feed mix samples and for all 4 AFs in the peanut and feed mix samples were in or close to the acceptable range (66 – 86%). Unfortunately, the recovery rate of OA from the peanut samples was very low (12-14%), therefore the samples with no detectable OA were reported as 'less than 1.5 µg/kg' (the approximate correction of 0.2 µg/kg at 13% recovery).

None of the Point of Sale samples of branded and non-branded feed mix (Table 11.1) contained detectable AF residues. Two branded feed mix samples exposed to climatic conditions for 1 month, however, had low levels (<0.8 μg/kg) of AFB_1. One non-branded feed mix sample and 1 branded feed mix sample exposed for 2 months also had low levels (<0.4 μg/kg) of AFB_1.

None of the Point of Sale samples of branded feed mix were found to contain detectable OA residues, but 1 of the Point of Sale samples of non-branded feed mix showed a level of 20 μg/kg of OA. One of the non-branded feed mix samples exposed to climatic conditions for 1 month had a level of 4.7 μg/kg OA and 1 of the branded feed mix samples exposed for 2 months had a level of 1.9 μg/kg OA, but all other exposed feed mix samples had concentrations of OA less than the detectable limits of 0.2 μg/kg.

Some Point of Sale and exposed samples of both branded and non-branded peanuts had detectable levels of AFB_1 and AFB_2 (Table 11.2). In the branded peanuts, these levels were all quite low (< 1.5 μg/kg), the highest occurring in a sample that had been exposed to climatic conditions for 1 month. There was no clear temporal pattern to the AF levels in the branded peanut samples, with similar concentrations occurring in 1 Point of Sale sample and in some feeder samples that had been exposed for 30, 60 and 90 days.

Nearly all of the non-branded peanut samples had detectable levels of AFB_1 and several also had detectable levels of AFB_2. Two of the non-branded peanut samples had AFB_1 that exceeded the MPL of 20 μg/kg: 1 of the Point of Sale samples contained 24.3 μg/kg AFB_1, whilst 1 of the samples exposed for 2 months contained 341.0 μg/kg AFB_1. Only 1 of the non-branded peanut samples exposed for 3 months contained an AF concentration over 0.2 μg/kg, and this was only 1.4 μg/kg AFB_1, so, as with OA, there was no clear temporal pattern to the presence of AF.

EXPERIMENT 2 - MAIN STUDY TO INVESTIGATE MYCOTOXIN PRESENCE AND PRODUCTION IN WILD BIRD FEED IN BRITAIN

The climatic variables measured in the spring, summer exposure and storage treatments are presented in Table 11.3.

Table 11.3: Environmental Temperature and Relative Humidity Range in the Treatment Categories.

Treatment	Average maximum temperature °C (range)	Average minimum temperature °C (range)	Average maximum relative humidity % (range)	Average minimum relative humidity % (range)
Spring Exposure	17.7 (9.0 - 27.0)	4.1 (0.0 - 14.0);	95.5 (80.0 - 100)	49.7 (9.0 - 29.0)
Summer Exposure	25.7 (9.2 - 21.4)	12.8 (5.0 - 10.0)	93.9 (9.0 - 78.0)	49.5 (41 - 67)
Storage	21.1 (6.0 - 17.0)	15.9 (7.8 - 21.1)	62.0 (2.5 - 53.0)	39.9 (31.0 - 52.0)

For the HPLC analyses, the recovery limits were within the acceptable range and the corrected results obtained are presented in Table 11.4 and 11.5.

Table 11.4: Concentration of Total AF and OA in each of the branded wild bird peanut products tested.

Product Type	Sample	Total AF Concentration (µg/kg)				OA Concentration (µg/kg)			
		Point of Sale	Spring	Summer	Storage	Point of Sale	Spring	Summer	Storage
Branded	1	<0.8	0.2	<0.8	<0.8	<0.2	<0.2	<0.2	<0.2
Branded	2	<0.8	<0.8	<0.8	<0.8	<0.2	<0.2	<0.2	<0.2
Branded	3	<0.8	0.5	<0.8	<0.8	<0.2	<0.2	<0.2	<0.2
Branded	4	575	<0.8	n.a.	<0.8	0.4	<0.2	n.a.	<0.2
Branded	5	695	435	325	580	0.3	<0.2	<0.2	0.2
Branded	6	<0.8	<0.8	<0.8	<0.8	<0.2	<0.2	<0.2	<0.2
Branded	7	<0.8	<0.8	0.6	<0.8	<0.2	<0.2	<0.2	<0.2
Branded	8	<0.8	<0.8	<0.8	0.2	<0.2	<0.2	<0.2	<0.2
Branded	9	900	6.9	500	3070	<0.2	<0.2	<0.2	<0.2
Branded	10	<0.8	0.2	<0.8	0.2	<0.2	<0.2	<0.2	<0.2
Branded	11	0.7	0.6	0.9	0.9	<0.2	<0.2	<0.2	<0.2
Branded	12	0.6	270	<0.8	195	<0.2	<0.2	<0.2	<0.2
Branded	13	0.2	3.5	0.2	0.2	<0.2	<0.2	4.4	<0.2
Branded	14	<0.8	0.2	0.3	0.3	<0.2	<0.2	<0.2	<0.2
Branded	15	<0.8	0.3	0.4	5.5	<0.2	<0.2	<0.2	<0.2
Branded	16	64	68	125	18.3	<0.2	0.5	<0.2	<0.2
Branded	17	0.2	0.2	<0.8	0.3	<0.2	<0.2	<0.2	<0.2
Branded	18	<0.8	0.3	0.2	0.4	<0.2	<0.2	<0.2	<0.2
Branded	19	<0.8	0.2	0.2	0.3	<0.2	<0.2	<0.2	<0.2
Branded	20	<0.8	<0.8	<0.8	0.2	<0.2	48.1	<0.2	<0.2

*Limit of quantification was 0.2 µg/kg for AFB_1, AFB_2, AFG_1, AFG_2 and OA; ** n.a. = sample not available.

Table 11.5: Concentration of Total AF and OA in each of the non-branded wild bird peanut products tested.

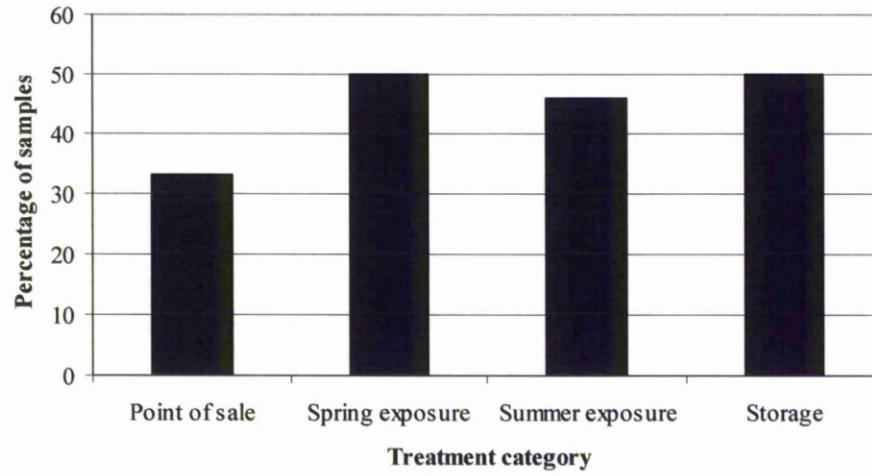
Product Type	Sample	Total AF Concentration (µg/kg)				OA Concentration (µg/kg)			
		Point of Sale	Spring	Summer	Storage	Point of Sale	Spring	Summer	Storage
Non-branded	21	1.5	270	400	1.2	<0.2	<0.2	<0.2	0.3
Non-branded	22	<0.8	<0.8	<0.8	<0.8	<0.2	<0.2	<0.2	0.2
Non-branded	23	<0.8	<0.8	<0.8	<0.8	0.3	<0.2	<0.2	0.2
Non-branded	24	<0.8	<0.8	0.2	<0.8	<0.2	<0.2	<0.2	<0.2
Non-branded	25	<0.8	<0.8	0.2	<0.8	<0.2	<0.2	<0.2	<0.2
Non-branded	26	0.7	<0.8	0.4	0.3	<0.2	<0.2	<0.2	<0.2
Non-branded	27	<0.8	<0.8	<0.8	<0.8	<0.2	<0.2	<0.2	0.2
Non-branded	28	<0.8	<0.8	<0.8	<0.8	<0.2	<0.2	<0.2	<0.2
Non-branded	29	<0.8	<0.8	0.2	<0.8	0.2	<0.2	<0.2	<0.2
Non-branded	30	<0.8	<0.8	0.2	<0.8	0.2	<0.2	<0.2	<0.2
Non-branded	31	<0.8	<0.8	<0.8	<0.8	<0.2	<0.2	<0.2	<0.2
Non-branded	32	<0.8	0.4	<0.8	<0.8	<0.2	<0.2	<0.2	<0.2
Non-branded	33	<0.8	<0.8	<0.8	<0.8	<0.2	<0.2	<0.2	<0.2
Non-branded	34	1.5	190	0.2	<0.8	<0.2	<0.2	<0.2	<0.2
Non-branded	35	6	0.2	<0.8	0.2	<0.2	0.4	<0.2	<0.2
Non-branded	36	<0.8	<0.8	<0.8	<0.8	<0.2	0.2	<0.2	<0.2
Non-branded	37	2.4	1.6	1950	3.4	<0.2	<0.2	<0.2	<0.2
Non-branded	38	<0.8	<0.8	<0.8	<0.8	<0.2	0.2	<0.2	<0.2
Non-branded	39	<0.8	0.2	<0.8	0.3	<0.2	<0.2	<0.2	<0.2
Non-branded	40	<0.8	<0.8	<0.8	0.2	<0.2	0.2	<0.2	<0.2

*Limit of quantification was 0.2 µg/kg for AFB_1, AFB_2, AFG_1, AFG_2 and OA

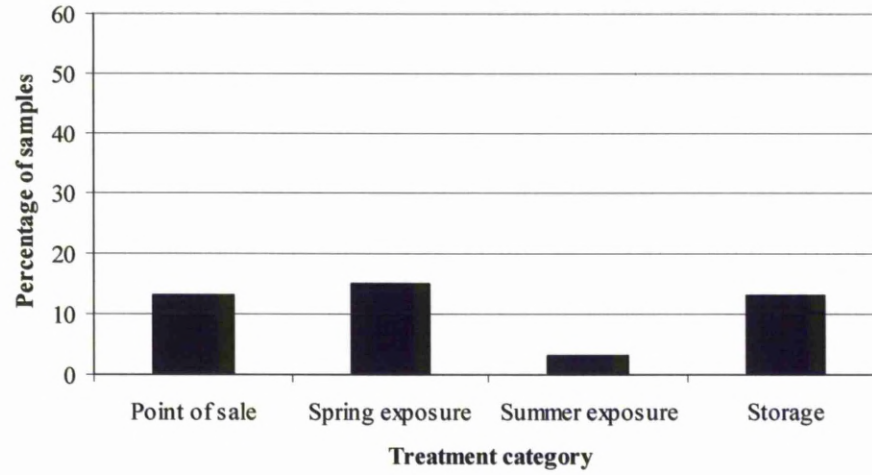
The number of samples with detectable mycotoxin residues ranged from 13-20 (32.5 - 50%) for each of the treatment categories for AF and 1-6 (2.5 - 15%) for OA (Figure 11.2).

Figure 11.2: Percentage of samples that contained (a) detectable AF residues and (b) detectable OA residues.

(a)



(b)



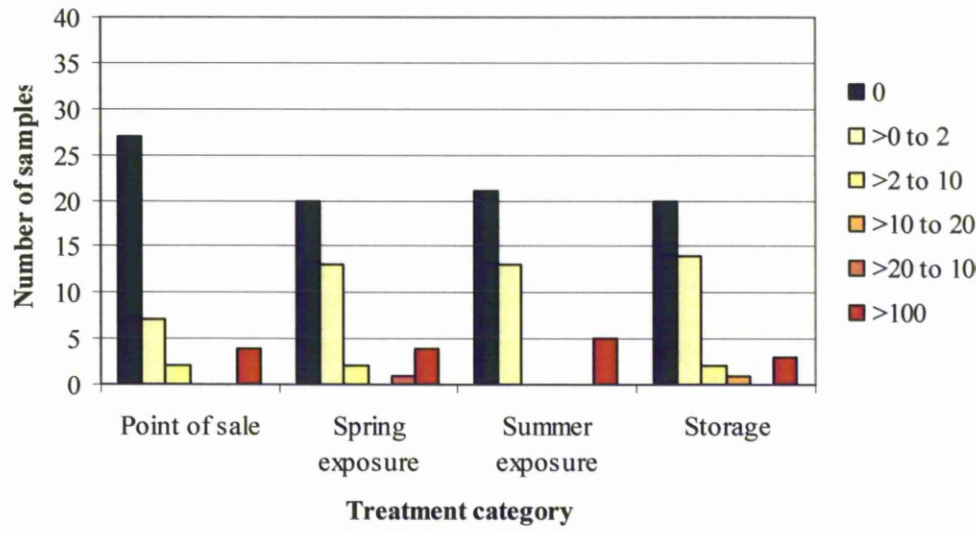
The number of treatment categories that had detectable levels of AF was summarised for each of the 40 peanut products to gauge the heterogeneity in the results obtained. Twenty-five per cent (10/40) of peanut products tested were negative for AF in all 4

treatment categories, 40% (16/40) of products were positive for AF in 3 or 4 of the treatment categories and 75% (30/40) products tested positive for AF in at least 1 treatment category. Eight peanut products had detectable AFB_1 levels exceeding 20 μg/kg in 1 or more of the treatment categories; 7 of which had detectable AF levels in 3 or 4 of the treatment categories. In contrast, 63% (25/40) of the peanut products tested were negative for OA in all treatment categories and the remaining 37% (15/40) had detectable OA levels in 1 or 2 of the treatment categories only.

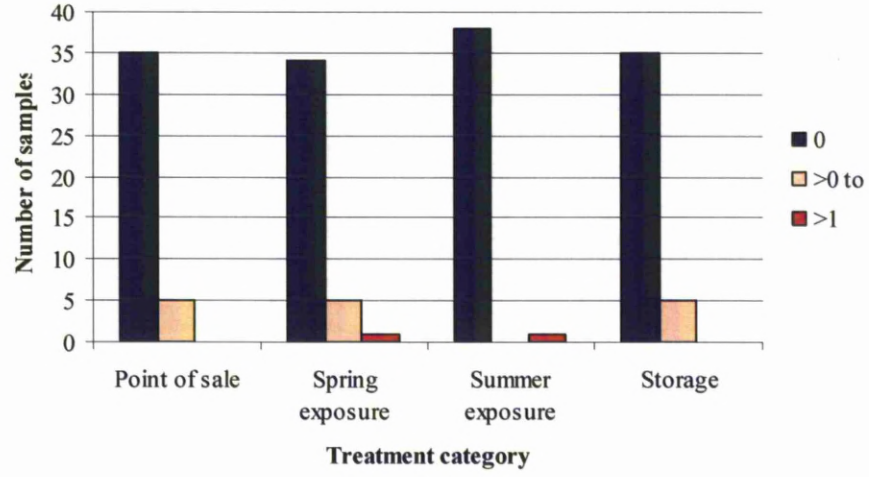
Total AF and OA concentrations for all the peanut products in each of the treatment categories are shown in Figure 11.3. AFB_1 and total AF concentrations ranged from 0-2570 and 0-3180 μg/kg respectively across all the products and treatment categories. For each of the treatment categories, between 3 and 5 of the products contained AFB_1 above the MPL of 20 μg/kg; across the treatment categories 16 of the 17 products that exceeded the MPL had $AFB_1 > 100$ μg/kg.

Figure 11.3: (a) Total AF concentration µg/kg and (b) OA concentration µg/kg for all peanut samples for each of the treatment categories.

(a)



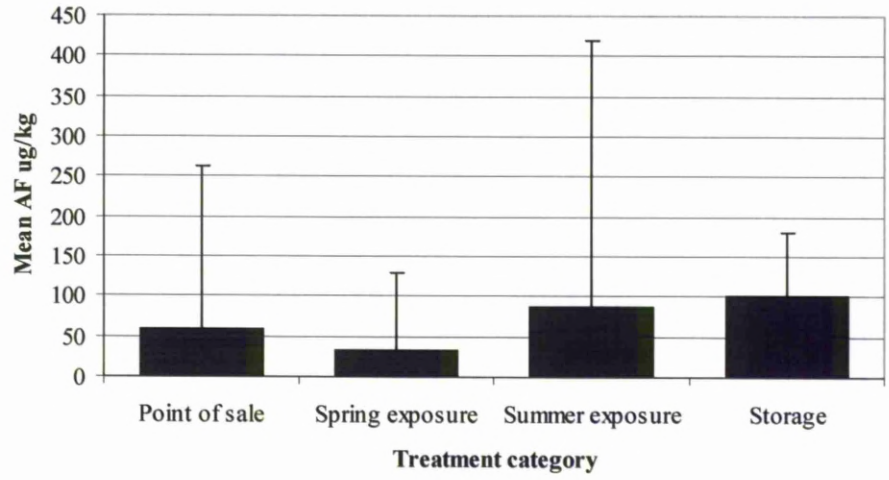
(b)



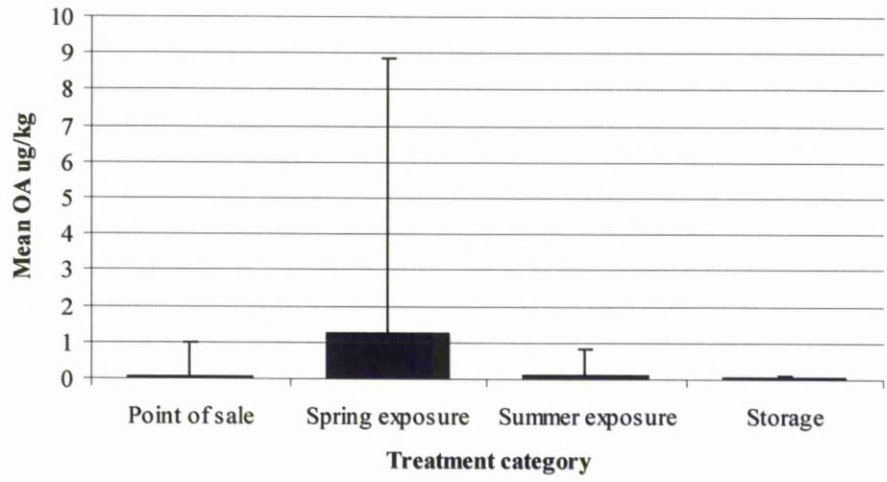
The mean and standard deviation for total AF and OA concentration for each of the treatment categories is shown in Figure 11.4 and indicates the high degree of variability in the absolute concentrations of mycotoxin obtained by the HPLC analyses.

Figure 11.4: (a) Mean total AF concentration µg/kg and (b) mean OA concentration µg/kg for all peanut samples for each of the treatment categories. Error bar represents standard deviation.

(a)



(b)



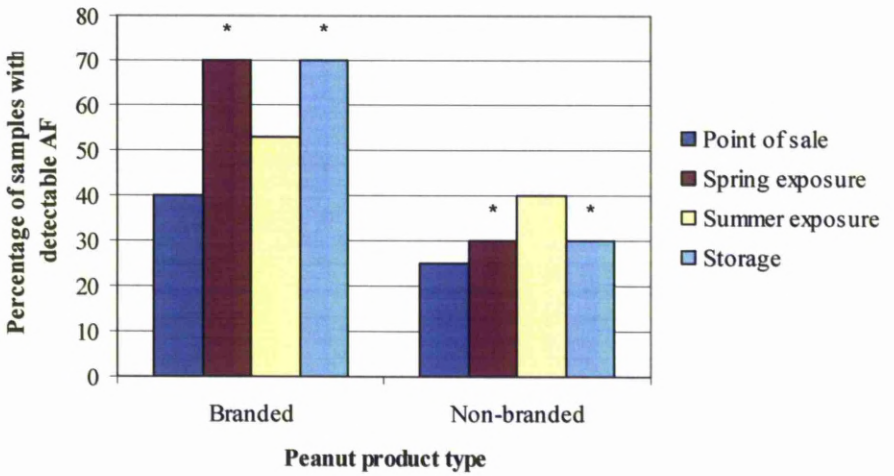
There was no significant difference between the paired data comparing the Point of Sale treatment results, as a baseline control, with the Spring Exposure treatment (t_s=-0.644, P=0.520), Summer Exposure treatment (t_s=-0.508, P=0.612) or with the Storage

treatment (t_s= ¯0.366, P=0.715) results, using the Wilcoxon Signed Rank Sum Test for non-parametric data. It is interesting to note that the 2 samples with total AF> 1000 μg/kg were in the Summer Exposure and Storage treatment categories and that the 2 samples with OA> 1 μg/kg were in the Spring Exposure and Summer Exposure treatment categories; none of the peak values of AF or OA were within the Point of Sale sample group.

Qualitative comparison between branded screened peanuts and non-branded peanuts, using the Pearson chi-square test and binary data based on AF levels above the limit of detection found no significant variation in the Point of Sale (X^2=1.026, P=0.311) or Summer Exposure (X^2=6.26, P=0.429) treatments but significant variation at the 95% confidence limit for the Spring Exposure (X^2= 6.40, P=0.011) and Storage (X^2= 6.40, P=0.011) treatments, AFs being more frequently detected in the branded group for each of these latter treatment categories (Figure 11.5a). Quantitative comparison between the same groups within each treatment category using the Mann Whitney-U test and absolute mycotoxin concentrations also found no significant variation for the Point of Sale (t_s= 169.5 P=0.321) or Summer Exposure (t_s=157.0, P=0.310) treatments, but did find significant variation for the Spring Exposure (t_s=123.0, P=0.026) and Storage (t_s=111.0, P=0.010) treatments (Figure 11.6a), with AF levels higher in branded *vs* non-branded peanut products. Qualitative (Figure 11.5b) and quantitative (Figure 11.6b) comparisons of the OA data identified no significant difference between the branded and non-branded peanuts for any of the treatment categories.

Figure 11.5: Proportion of samples that contained (a) Detectable AF residues and (b) Detectable OA residues. * denotes significant difference between branded and non branded peanuts with 95% confidence, using Pearson chi-square non-parametric test.

(a)



(b)

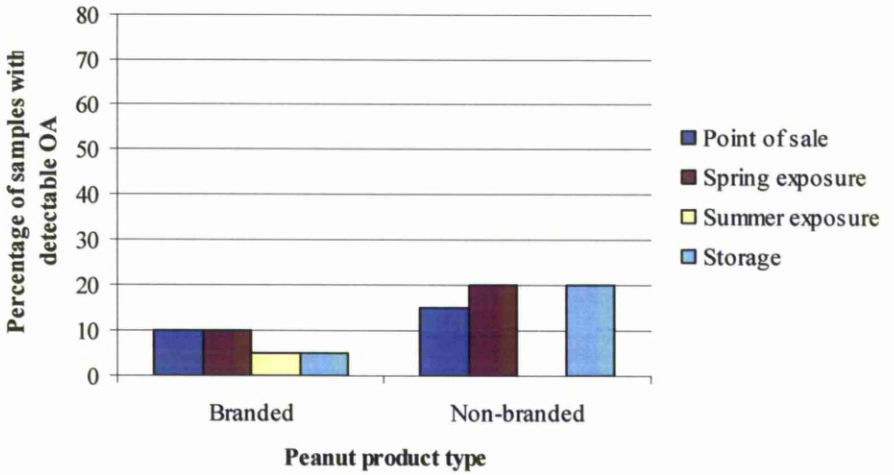
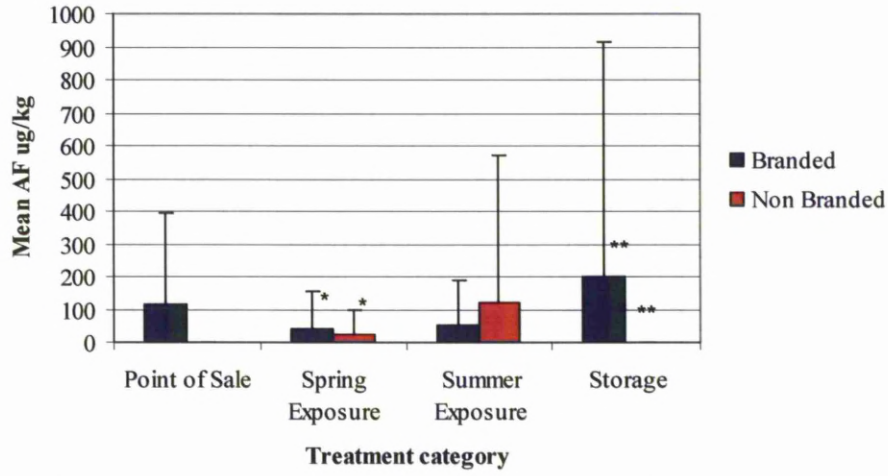
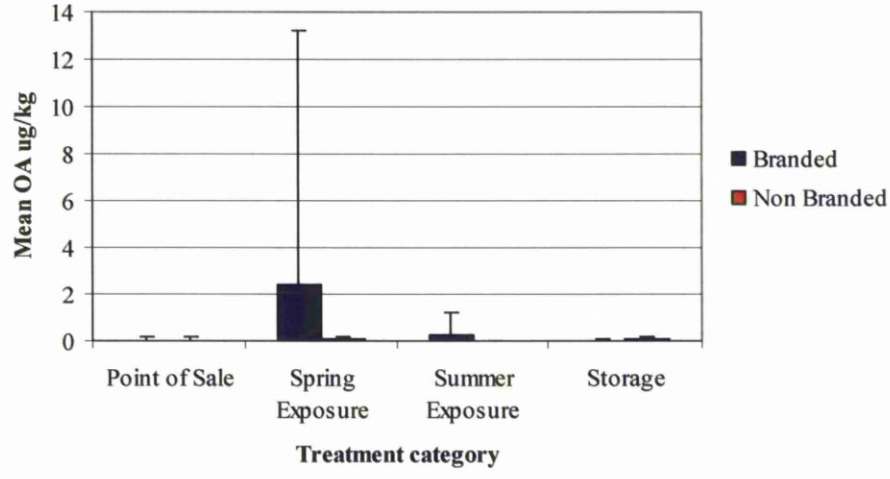


Figure 11.6: (a) Mean AF concentration µg/kg and (b) mean OA concentration µg/kg for all peanut samples for each of the treatment categories. Y-error bar represents standard deviation. * denotes significant difference between branded and non branded peanuts with 95% confidence, Mann Whitney U non-parametric test for independent samples.

(a)



(b)



The single peanut granule sample under the Summer Exposure treatment conditions formed a solid cake of food with visible fungal contamination of its core (Figure 11.7). Mycological examination of this sample using culture on Sabauraud's medium was

performed and an overgrowth of a *Mucor* sp. was isolated. Peanut granules within each of the treatment categories had detectable levels of AF although all were below 5 μg/kg. Only the Summer Exposure treatment sample had detectable OA and this contained the second highest level recorded in the study at 4.4 μg/kg.

Figure 11.7: Peanut granule sample following Summer Exposure treatment: (a) as removed from feeder and (b) on cut transverse section.

(a)

(b)

BIRD FEEDER RESIDUE SCREENING – PILOT SURVEY

Food residues collected from hanging feeders were submitted from 7 gardens in south-east England in August 2005. Detectable AF residues were found in all 7 samples, 2 of which greatly exceeded the 20 μg/kg MPL for AFB_1. Detectable OA residues were found in 2 samples, neither of which approached the 100 μg/kg EU guidance limit for OA in poultry foodstuffs. Further information on the heritage of the feeder residues is given in Table 11.6:

Table 11.6: AF and OA levels in Bird Feeder Residues (μg/kg)

Sample	AFB_1	AFB_2	AFG_1	AFG_2	Total AF	OA	Feeder type	Food type	Estimate time in feeder
1	0.9	<0.2	<0.2	<0.2	0.9	<0.2	Wire mesh	Unbranded seed & unbranded nuts	5 months
2	1.7	1.5	<0.2	<0.2	3.2	<0.2	Wire mesh	Branded peanuts	>1 year
3	0.7	<0.2	<0.2	<0.2	0.7	<0.2	Plastic seed hopper	Branded seed	3 months
4	7.4	1.5	6.1	1.5	16.5	1.0	Wire mesh & Plastic seed hopper	Branded seed & Unbranded peanuts	3 months
5	61,710	4,,810	39,850	2,730	109,100	<0.2	Wire mesh	Branded peanuts	3 weeks
6	690	28.2	181	11.2	910	<0.2	Plastic seed hopper	Non branded seed	4 months
7	4.2	0.5	1.1	<0.2	5.8	2.6	Wire mesh	Branded peanuts	6 months

QUESTIONNAIRE OF GARDEN BIRD FEEDING PRACTICE

Of the 251 respondents (from all members of staff on the RSPB and ZSL email lists, total number unknown), 130 (52%) fed branded peanuts and 78 (31%) fed non-branded peanuts; 168 (67%) of the 251 respondents fed branded seed and 70 (28%) fed non-branded seed. Out of 251 respondents, 154 (61%) fed garden birds all year round, compared with 58 (23%) who provided feed during winter months only (November to March). When asked whether they emptied and refilled feeders or just topped them up when necessary, 98 (39%) out of 251 said they topped feeders up, 61 (24%) said they emptied and refilled feeders each time, and the remaining 91 (36%) said they did a mixture of the 2 (1 person did not answer this question).

12.4 DISCUSSION

Samples of garden bird food products were found to contain AF and OA, with both branded and non-branded products positive for residues. Peanut products showed the highest prevalence and intensity of contamination whilst seed products, containing predominantly mixed seed and cereal, had few positive samples for AF or OA in the

pilot study and, where toxins were detected, the levels were relatively low. The need for further assessment of AF levels in seed products for wild bird consumption, for example sunflower seed, has been raised by the EC (2006), however, the main study considered peanuts alone as the apparently higher risk product.

Whilst problems with the extraction process for OA in the pilot study for peanut samples complicated direct comparison of results between the food types screened, the extraction process was successful within acceptable guidelines for all samples within the main study where AF were more frequently detected than OA in the peanut samples. Although OA is considered a storage toxin produced under temperate climate conditions, no samples across the 4 treatment categories had OA levels that approached the poultry feedstuffs guidance limit of 100 μg/kg OA.

The pilot study results showed a high degree of heterogeneity in the AF and OA results obtained from single food sources. For the main study, results were found to be relatively consistent for the majority of peanut samples across the 4 treatment categories: 40% of peanut samples had no AF detectable residues in any of the 4 treatment categories and 25% of peanut samples had detectable AF residues in 3 or 4 of the treatment categories. This indicates that whilst heterogeneity was present in each peanut sample, AF contaminated products were not the result of an isolated pocket of mycotoxin production.

AFs were detected in 42% (71/160) of peanut samples in the main study; 11% (17/160) of which were above the MPL and 10% (16/160) of which exceeded 100 μg/kg, a level shown to cause 47% mortality in northern cardinals (*Cardinalis cardinalis*) when fed on such a diet for 3 weeks (Henke et al., 2004). In the pilot study, 1 of the non-branded peanut samples had an AF concentration above the MPL at Point of Sale and 1 of the non-branded samples exposed to the British climate for 2 months exceeded 100 μg/kg AF.

The pilot study revealed no clear temporal pattern to the AF or OA levels exposed to the British climate for up to 3 months, due to heterogeneity of mycotoxin distribution within the Point of Sale samples. In the main study, the protocol was refined to compare AF and OA levels exposed to the British climate for a period of 1 month only under different weather conditions (Spring Exposure or Summer Exposure), rather than a variable duration (1-3 months) for each of the forty independent suppliers. No statistically significant evidence to support AF or OA production under British climatic conditions or in storage was found in the main study, however, this does not preclude the possibility that this occurs. Oberheu et al. (2001a,b) found relative humidity (R.H.) to be the only climatic predictor of AF and OA concentrations in grain samples within game bird feeders; evaluation of any relationship between R.H. and mycotoxin levels was not possible with the climatic data collected in this study. It is interesting to note that the peak AF and OA values recorded were in samples from the climate exposure and storage treatments and not in the Point of Sale samples. Indeed, the study of food residues from garden bird feeders in south-east England found 2 samples with extremely high AF residues, providing anecdotal evidence for AF production in the feeder since such high levels are unlikely to have been present at the point of sale.

The pilot study found a greater number of the non-branded peanut products positive for AFs than the branded peanut products; also, the greatest absolute levels were found in non-branded products, 2 of which exceeded the AFB_1 MPL. This is consistent with the hypothesis that mycotoxin residues are more frequently present in non-branded products, for which there are no specified quality control procedures, compared to branded products which have been screened for AF levels. To further test this hypothesis, a larger study was conducted in which the sample size was increased to 40 products. Twenty branded and 20 non-branded peanut products were obtained from independent suppliers and the AF and OA levels were compared. This larger study found no evidence to support the hypothesis that AF or OA residues were less frequently detected, or found at lower levels, in branded products than in non-branded products as there was no

significant difference among the point of sale samples. Conversely, results from the Spring Exposure and Storage treatments support the reverse hypothesis. Given the degree of variation in the dataset and the contrasting results between the Spring and Summer Exposure treatments, however, consideration of the entire data set does not support a significant difference in mycotoxin levels between the branded and non-branded products.

There were many unknowns about the products used within this study and future research should include consideration of potentially confounding variables (e.g. packaging type, cost, retail source, year of harvest). The label information on quality control used in the branded products was frequently unclear and needs to be improved. There is currently no legal requirement for the packaging of food products for wild bird consumption to include a Best Before Date, although the addition of advice on storage and product shelf life on the product label might be worthwhile. Whilst no quality control information was available for the non-branded peanuts, the absence of information does not indicate that AF screening had not been performed. Nevertheless these results highlight a need for the garden bird food trade to review quality control procedures for AF screening.

The results of this study indicate that supplementary food for wild birds in Great Britain can be a source of mycotoxins; whilst the OA levels obtained did not approach the concentrations shown to cause adverse affect to birds in experimental studies, in some cases the AF concentrations were in the range known to cause acute or chronic toxicity in some bird species. Synergism of mycotoxin exposure can occur, and other factors relevant to winter feeding of garden birds, for example poor nutrition and low ambient temperature, can exacerbate their toxic effects (Huff et al., 1981).

Mechanical damage to the food product can increase the likelihood of mycotoxin production (Santin 2005). Milling of peanuts to produce granules may mimic this effect and, given the marked fungal growth that occurred on the single sample of peanut

granules in this study, further research is required to evaluate mycotoxin contamination of these products.

The importance of agricultural crops or wild seed as a source of AF or OA exposure for wild birds requires evaluation in Great Britain. In the U.S.A., studies of corn left in the field after harvest have found levels of AFB_1 up to 5000 μg/kg (Couvillion et al., 1991) and 1,210,000 μg/kg (Stewart 1985) but no detectable OA levels (Couvillion et al., 1991).

To examine the differential contributions of wild versus supplemental seed as sources of AF to wild birds, a study was performed in Oklahoma, U.S.A., on bobwhite quail killed by hunters. The oesophageal contents from these birds were classified according to their seed type (wild seed, supplemental seed, mixed food). AF levels found in the wild seed component of the birds' diet were greater than those for the supplementary seed, although mean AF concentrations were below 2.5 μg/kg. This result was unexpected since much higher levels of AF had been detected in supplementary feed samples taken directly from feeders available to the birds over the period of the study. This finding prompted the authors to suggest that birds may have the capacity to discriminate against contaminated food sources (Oberheu et al., 2001a). Subsequently, an experimental study of 3 species of wild-caught birds found that bobwhite quail (*Colinus virginianus*) and white-winged doves (*Zenaida asiatica*) did not discriminate against AF-contaminated food; in the same study green jays (*Cyanocorax yncas*) were able to discriminate contaminated food sources although they consumed the same overall amount of contaminated food over the 72 hour trial as the other species (Perez et al., 2001).

Peanuts are a common supplementary food product for garden birds; c.80% of British Trust for Ornithology (BTO) Garden BirdWatch participants offer peanuts to the wild birds in their garden in the winter and c. 73% do so in the summer (BTO, *unpublished data*). The results of this questionnaire survey found that a greater percentage of respondents provided branded peanuts and branded seed than non-branded products of

either food type. It is important to note, however, that ZSL and RSPB staff might be more aware of best feeding practices for garden birds than the general public and their responses might not be representative of anthropogenic garden bird provisioning in general. Nevertheless, around one third of respondents provision garden birds with non-branded food products for which there is no information on quality control procedures.

The majority of questionnaire respondents provide food for garden birds year-round. Sweeney et al. (1998) have shown that production of AF and OA occurs under ambient temperature conditions similar to those likely to occur during the warmer summer, but not the cooler winter months, in Great Britain. It is possible that the move to summer-feeding of garden birds might be associated with an increased risk of mycotoxin production in supplementary food products, particularly during periods of warm, wet weather.

In an RSPB survey of the general public, 23% of respondents never cleaned their hanging feeders and in this questionnaire survey, only one quarter of respondents emptied bird feeders before refilling them. Topping up the contents of a feeder may lead to residues at the base of the feeder remaining *in situ* for some time. The current study found detectable, and sometimes high, levels of AF in feeder residues: consequently feeders should be emptied and cleaned before being refilled.

Taken together, the findings of this series of studies indicate that current practice at feeding stations is suboptimal and might increase the risk of mycotoxin, in particular AF, exposure to garden birds from supplementary feed. Assessment of the pathological significance, if any, of AF exposure to British garden birds remains a research priority. One way to do this would be through a series of experimental exposure studies, although this would be expensive and the results would have to be regarded as specific-specific. Alternatively, detailed post mortem investigations, including histopathological and mycotoxin examinations, of wild garden birds found dead would provide information on exposure levels amongst species and seasons and would help to detect overt signs of

toxicity or associations between the presence of mycotoxins and the presence of other conditions, such as infectious disease.

CHAPTER 12: GENERAL DISCUSSION

Dynamic state of endemic and emergent disease threats

In this thesis, the findings of surveillance of garden bird mortality across Great Britain, achieved through 2 independent schemes using opportunistic and systematic methodologies (collectively called the Garden Bird Health *initiative* (GBH*i*)), are presented. Despite the restricted time of study, over a 3-year period only, the patterns of infectious disease within wild bird populations have been found to be highly complex and dynamic. Major changes have occurred in the epidemiology of some of the best-characterised bacterial, parasitic and viral pathogens of garden bird species between 2005 and 2008.

i. Bacterial

Salmonellosis is endemic in Great British passerine populations and is known to be seasonal, chiefly occurring during the winter months. Disease incidents are typically observed affecting gregarious granivorous passerines in the vicinity of garden feeding stations (Pennycott et al., 2006). The findings in this study support these observations from the published literature. However, interrogation of retrospective data for the 11-year period preceding the GBH*i* has identified previously unrecognised temporal and spatial trends of the *S.* Typhimurium phage types responsible for salmonellosis in passerines in England and Wales. The overall number of salmonellosis incidents has declined sharply across Great Britain between 2005 and 2008 and, although the drivers of this trend are incompletely understood, this represents a significant change in the occurrence of this endemic pathogen.

ii. Parasitic

Trichomonosis has been a well-recognised endemic disease of columbiform species, and of raptors that predate or scavenge on infected pigeons and doves, since historical times; this is reflected in the colloquial names of 'canker' used by pigeon fanciers and 'frounce' used by falconers for the disease in these bird groups respectively (Forrester et al., 2008). *T. gallinae* infection in British columbiform species is known to cause

isolated cases and localised outbreaks of disease (Cousquer 2003). A recent survey of feral pigeons (*Columba livia*), contemporaneous with this study, found high rates of asymptomatic *T. gallinae* carriage consistent with hyperendemic infection in London (Jenkins 2007). The seasonal epidemic finch mortality that occurred in the late summer months of 2006 and 2007, and subsequent greenfinch (*Carduelis chloris*) and chaffinch (*Fringilla coelebs*) population declines attributed to trichomonosis, are unprecedented in their scale for British wild birds. Molecular epidemiological investigations performed in this study support spill-over of parasite infection from sympatric columbiform species to greenfinch and chaffinch populations in, or shortly before, spring 2005, as the most plausible origin of this emerging infectious disease of Fringillidae. Following the spread of finch trichomonosis to southern Fennoscandia in summer 2008, further mortality incidents have occurred in continental Europe in 2009 with an extended range in Fennoscandia (Ytrehus 2009), confirmed outbreaks in Germany (Peters et al., 2009) and suspected incidents in Denmark (Ytrehus 2009). Trichomonosis should be considered a serious threat to Fringillidae populations in continental Europe and, based on observations from Great Britain in this study I predict further large-scale mortality might occur in the summer months of 2010.

iii. Viral

Avian pox infection is typically associated with sporadic disease affecting individual birds, most commonly the wood pigeon (*Columba palumbus*), dunnock (*Prunella modularis*) and house sparrow (*Passer domesticus*) in Great Britain, although a diversity of species are known to be affected (Pennycott 2003; van Riper et al., 2007). This viral pathogen is considered endemic in these populations and affected birds are frequently observed with relatively minor lesions, which are frequently self-limiting, as with poultry species (van Riper et al., 2007). Since 2007, a novel avian pox infection affecting Paridae species, principally great tits (*Parus major*), has occurred in southern England. Infection is unusual in the severity of the disease with florid lesions that significantly impair vision or locomotion, predisposing the birds to predation or secondary infection with a high case fatality rate. The number of avian pox incident reports in Paridae species has continued to increase in 2008 and 2009 (GBH*i*,

unpublished data). Molecular epidemiology is required to determine whether this emerging infectious disease of Paridae is caused by a novel avian poxvirus strain. Alternative explanations for the increase in great tit cases should be explored, for example host susceptibility or change in the populations' of biting-insect vectors.

These findings underline the importance of long-term surveillance schemes, rather than isolated studies over restricted time frames, with robust methodologies to constantly re-inform our understanding of endemic, epidemic and emerging disease threats to native wild bird populations.

Benefits of wildlife disease surveillance

Effective surveillance is important to identify disease threats that impact animal welfare, species conservation and biodiversity, and that pose a threat to livestock, companion animal or public health. This multi-disciplinary study has reached conclusions and produced outputs that are relevant to each of these motivations.

i. Animal welfare

In this study, infectious disease accounted for a significant proportion of mortality in passerine species that visit garden feeding stations which will have adversely impacted their welfare. Feeding garden birds is now a common year-round hobby with the public adopting a responsibility and stewardship for the wild birds for which they provide. The provision of food to garden birds is practised on a large scale across Great Britain and influences their congregation (e.g. numbers of birds, species complement) at feeding stations, which might alter the dynamics of disease transmission (Kirkwood 1998; Robb et al., 2008). Poor feeder hygiene might promote exposure to faeco-orally transmitted bacterial pathogens. In addition, this study has highlighted the potential for mycotoxins in bird food to negatively impact garden bird health. Public information on best practice for feeding garden birds was published by the GBH*i* in 2005 in the form of leaflets and booklets which include recommendations on how to minimise the risk of disease events (e.g. hygiene precautions, rotating feeding sites, regular provision of clean water, use of high-quality food sources) affecting garden birds. The results of this study have been

used to create GBH*i* disease factsheets available online (GBH*i* 2005) which include pathogen-specific advice on prevention and control, along with information pertinent to public and companion animal health. These have been regularly updated and widely disseminated to the general public through collective press releases, ornithological non-governmental organisations, government bodies and the garden bird food industry.

Detailed information on feeding practice (e.g. type and volume of food, type and number of feeders, hygiene practises) has been collated through the systematic surveillance performed by the Garden BirdWatch (GBW) network and, in collaboration with the British Trust for Ornithology (BTO), we have recently undertaken analyses to identify anthropogenic risk factors for salmonellosis and trichomonosis. The intended output is to offer science-based guidance on mitigation measures to minimise risks of disease transmission between birds visiting garden feeding stations in order to improve their welfare.

Animal welfare is the principal concern of wildlife hospitals and rehabilitation centres and large numbers of wild animal casualties of a wide range of species are cared for at these centres each year. Outputs from this study were used to inform members of the British Wildlife Rehabilitation Council of the importance of infectious disease in passerine casualties and the emergence of trichomonosis (Lawson 2006a; Anon. 2007). In particular the range of wild bird species susceptible to *T. gallinae* infection and the importance of biosecurity, most specifically separate housing of passerine fledglings during the rearing season, was emphasised to help avoid nosocomial infection and spill-over of *T. gallinae* infection to novel avian hosts.

ii. Species conservation and biodiversity

This study has confirmed that the 2006 finch trichomonosis epidemic had a dramatic negative impact on breeding populations of British greenfinches and chaffinches over a short period. Seasonal mortality due to trichomonosis continues to occur, chiefly in these finch species; current population monitoring data from the BTO GBW, and the results of the BBS 2008 (Risely et al., 2009), indicate that further declines have occurred although

robust analyses are required to determine the relative rates of decline and how these are changing across Great Britain in time and space. The importance of continued surveillance to monitor cases of trichomonosis in biodiversity action plan-listed passerine species of conservation concern (e.g. house sparrow, bullfinch (*Pyrrhula pyrrhula*), yellowhammer (*Emberiza citrinella*) has been highlighted. Appraisal of the impact that finch trichomonosis may have on raptor populations is challenging since the carcasses of these cryptic and camouflaged species are inconspicuous. The garden-based citizen science network in this study does not offer the best opportunity to monitor disease in raptor populations. I am currently collaborating with the Hawk Conservancy Trust to investigate an apparent increase in the number of bird of prey casualties that they have received with trichomonosis since summer 2009 (K. Kirkbride, *pers. comm.*). Available hospital case records (species, annual and seasonal trends of submission, geographical distribution) from the Trust will be reviewed to evaluate the strength of evidence to support the perceived increase in raptor cases and whether a link with finch trichomonosis is plausible. For instance, predatory birds chiefly reliant on passerine prey (e.g. sparrowhawk (*Accipiter nisus*)) would be predicted to be more frequently exposed to *T. gallinae* infection than species which principally take small mammals (e.g. kestrel (*Falco tinnunculus*)). Raptor trichomonosis cases that result from finch predation would be predicted to peak in the late summer and autumn months in the period following greatest passerine mortality. Sequence analysis of the Fe- hydrogenase gene of *T. gallinae* isolates collected from a variety of raptor species is required to determine whether variation is present in the parasite strains and, if so, whether this indicates that these birds were infected as a result of predating or scavenging Columbidae or Fringillidae prey.

iii. Livestock and companion animal health

Wildlife populations are frequently cited as potential reservoirs of livestock disease (Simpson 2002). Whilst transmission of infection between captive poultry and wild bird populations is plausible for several of the bacterial, parasitic and viral pathogens diagnosed as a cause of morbidity or mortality during this study, the data do not support a conclusion that garden birds constitute a particularly significant source of infection, for

example infection with *S.* Typhimurium definitive phage type (DT)40 and DT56(var) (Pennycott et al., 2006).

Since 2005, the threat of highly pathogen avian influenza (HPAI) H5N1 incursion to the UK, and the role which wild birds might play in this regard, has been of significant concern with particular attention paid to mortality reports of waterfowl and gull species. Garden birds are not considered a high risk group for detection of the incursion of HPAI H5N1 infection in Great Britain. Nevertheless governmental surveillance schemes for this notifiable disease of poultry based on reporting of wild bird carcasses benefited from an understanding of the results of this study. Information on the species and regions affected by the trichomonosis epidemic in 2006 were communicated to government, along with the GBH*i* disease fact sheet on trichomonosis, in order to provide information online to educate and reduce public concern, and to avoid wasting government resources.

Whilst there is minimal evidence to indicate that garden bird disease frequently affects captive aviary birds or racing pigeons, the potential for transmission exists, for example with *T. gallinae* or avian pox infection. Specific advice has been communicated to specialist bird keepers as an output of this study recommending standard biosecurity precautions through articles in Cage and Aviary Birds magazine (e.g. West 2009).

iv. Zoonoses and public health

S. *Typhimurium* infection with wild-bird associated phage types has been shown to be a cause of disease in humans (Alley et al., 2002). In collaboration with the Health Protection Agency, the findings of this study support the hypothesis that garden bird populations are a source of this infection for humans in Britain. This underlines the importance of basic hygiene advice provided to all those who submitted carcasses for post mortem examination in this study (e.g. avoiding direct carcass handling, cleaning feeders outside the kitchen).

Conservation medicine approach

This study exemplifies the conservation medicine approach where a multi-disciplinary team undertakes wildlife disease investigation, incorporating contributions from veterinarians, ornithologists, medical reference centres, molecular diagnosticians, toxicologists, industry, government, members of the public and educated citizen science networks (Daszak et al., 2004) and, in turn, benefits each of these communities.

i. Wildlife veterinarians

This study benefited from the contributions of a number of veterinarians with an established interest in wild bird disease; this collaborative approach offered an opportunity to share findings and background knowledge. At the outset of this research, a standardised post mortem and microbiology protocol was developed which facilitated integration of results. Collaboration between veterinarians working with an established interest in the field, covering different regions, enabled an effective national surveillance scheme across Great Britain, with excellent geographical coverage. Interpretation of the significance of this data is far simpler than dealing with multiple regional reports on the same disease investigated with variable methodologies.

The collective fund-raising approach of the GBH*i* also gained credibility from the multiple collaborating organisations. Approaches to funding bodies with a 'single voice' helped avoid wasted resources with repetition of work through independent research groups competing for funding.

ii. Ornithologists

Collaboration with ornithologists has been fundamental to many findings in this thesis. Modelling of population monitoring data from garden habitats, and of breeding bird populations, was required to assess the population impact of trichomonosis in British greenfinches and chaffinches. Ring return data was used to investigate and identify migratory bird species that are the most plausible candidate for vectors of *T. gallinae* transfer to continental Europe. Ornithological data on species' population distributions has been compared with the distribution of disease incidents for particular pathogens; for

instance the matching distribution of *E. coli* serotype O86 infection and siskin populations indicates that this species might play a key role in the epidemiology of this disease. Ornithologists' input on natural history, such as feeding and breeding behaviours and dominance hierarchies at feeding stations, has helped inform discussion of findings, for example on reasons why some species or sexes may be more frequently exposed or predisposed to infection with certain pathogens.

iii. Medical reference laboratories

Collaborative approaches between veterinary and medical communities are required to investigate the role of wildlife as sources of zoonotic infection, for example the postulated link between garden bird and human salmonellosis with *S.* Typhimurium DT40, DT56(var) and DT160 identified in this study. Collaboration with medical molecular diagnosticians with a research interest in protozoan parasites at the University of East Anglia (UEA) has shown that the finch *T. gallinae* strain studied from Great Britain appears to have a clonal origin.

iv. Toxicologists

Toxicologists at the Food and Environment Research Agency have assisted with this research on testing wild bird tissue and food products for mycotoxin residues. These findings indicate that garden birds in Britain are exposed to aflatoxins although future work combining histopathological appraisal in combination with tissue residue analyses is required to determine aflatoxins' pathological significance, if any.

v. Garden bird food industry

Safe levels of aflatoxin residues within peanuts marketed for wild bird consumption has been an important issue for the bird care industry in recent years, being incorporated in the industry standards for the Birdcare Standards Association (BSA 2009). Results of this study help to fill the knowledge gap reported by the 2002 government agency consultation (FSA 2003) that concluded that negligible scientific data was available to evaluate the importance of mycotoxin exposure in wild birds.

The garden bird food industry provided funding for this study, and attended regular update meetings on findings, but did not directly contribute to the scientific content or research questions; consequently no conflict of interest has arisen. The study benefited from a close working relationship with the industry which offered access to information on historical trends for garden bird feeding. Outputs from this study have highlighted the importance of best practice and hygiene recommendations; the GBH*i* can accept some credit for the increase in hygiene-related products and best practice information available to the public in the marketing catalogues of many bird food companies over recent years.

vi. General public

The general public is highly motivated by animal welfare and native species conservation and are willing to report morbidity and mortality, not simply in response to a direct appeal for assistance but of their own volition when major disease events occur, exemplified by the marked increase in reports received during the first year of epidemic finch mortality due to trichomonosis in 2006. Whilst opportunistic reports from the public are vulnerable to bias, and are of variable quality, adoption of the incident definition for trichomonosis, informed by the findings of post mortem examinations, enabled us to use this dataset to identify spatial and temporal trends in the disease outbreak.

vii. Citizen science networks

Participants of the systematic surveillance scheme from the BTO's GBW uniquely provided us with a method to confirm absence of disease from monitored sites and to independently corroborate findings from the opportunistic scheme. Comparison of the perceived COD reported by GBW participants for each bird with the conclusions reached through post mortem examination has shown that the diseased birds were identified with high confidence but that infectious disease was an important contributory factor in many cases which had died of predation, which was under-appreciated by the GBW participants.

viii. Governmental bodies

Defra and Natural England have been updated with relevant findings throughout this study. As a direct consequence, the Institute of Zoology adopted chief responsibility for the investigation of passerine disease events for Defra in 2009 as part of the Great Britain Wildlife Disease Surveillance Partnership (Defra 2009).

Investigation of the epidemic mortality that occurred due to finch trichomonosis was facilitated since a collaborative research project was in place, in advance of the disease event, and could co-ordinate a response and investigate in real-time. This contrasts, for example, with the 2002 UK phocine distemper virus epidemic investigation, where the index case was confirmed and the UK disease outbreak began before the government-funded investigation was launched (Lawson et al., 2004).

Future development of the GBH*i* model

The combination of opportunistic and systematic surveillance methods used for disease surveillance in this study offers a useful model for work with other wildlife species in Great Britain and developed counties worldwide. This study had a positive high profile interface with the public, since it utilized an entirely non-invasive approach and provided a source of information and advice on garden bird health.

An extensive national sample archive for garden birds has been collated through this study that includes frozen and formalin-fixed tissue, frozen bacterial isolate libraries, cryopreserved trichomonad cultures, metazoan and protozoan parasite archives. This resource should support further pathogen-specific or species-specific research projects in the future. The sample archive collated since 1990 by the Cetacean Strandings Investigation Programme (CSIP) has fostered multi-disciplinary research on a variety of themes beyond disease, for example diet, age, and reproduction, and has contributed to over 150 publications in the peer-reviewed literature over this time (Deaville et al., 2009). The value of long-term wildlife tissue archives for toxicological investigations has also been shown (Jepson et al., 1999; Simpson et al., 2000; Walker et al, 2007). The

GBH*i* archive offers a unique resource which can be utilized in the future in a similar way.

i. Adaptive citizen science methodologies

Citizen science projects, such as the GBH*i*, use participant networks to investigate research questions on a large scale and real-life context. The logical extension to this approach is described as an adaptive citizen science project (Cooper et al., 2007) where the network manipulate their local environment with trial management options, following a randomized design, and continue to collect data to evaluate the effect of these actions. For instance, once risk factors for garden bird disease events are identified, the benefits and practicalities of mitigation management strategies (e.g. frequency of bird feeder cleaning) could be evaluated.

ii. Garden wildlife health surveillance

The GBH*i* provides a paradigm for health surveillance that could be applied to a variety of wildlife species that use garden habitats. A variety of native mammal and amphibian species of conservation concern regularly visit garden habitats, including UK priority species and those with dedicated biodiversity action plans (e.g. red squirrel (*Sciuris vulgaris*), hedgehog (*Erinaceus europaeus*), common toad (*Bufo bufo*)) (JNCC 2008). Whilst the reasons for species' declines are incompletely understood, there is a need for surveillance in order to confirm the relative importance of disease as a factor influencing these populations (Reeve 1994; Hof 2009). Examples of diseases that require further investigation include squirrelpox virus, adenovirus and rotavirus in the red squirrel (Tompkins et al., 2002; Duff et al., 2007: Everest et al., 2009), ranavirus infection and chytridiomycosis in Britain's amphibian population (Cunningham et al., 2005; 2007) and a number disease syndromes of unknown aetiology in hedgehogs (Plumb 2008). Species which are positively perceived by the public will be most amenable to study (e.g. red squirrel, amphibians), particularly those where there is some direct human-wildlife interface, for example maintenance of garden ponds or provision of food. A citizen science approach is likely to be unsuitable for assessment of disease in pest species, particularly rodents. Diurnal species will be most frequently observed; however,

studies involving some nocturnal species (e.g. hedgehog) seem practical. Collaboration between the stakeholder organizations and individuals with interests in each species or wildlife group, as in this study, will be critical to ensure maximum benefit from existing information and knowledge and to minimise costs by using all existing resources. The BTO GBW network has collected information on mammal species as part of the Tracking Mammals Partnership since 2004 (BTO 2008). A Reptiles and Amphibians in Gardens 2009 survey has recently been undertaken by the GBW network (BTO 2009). The BTO GBW offers the most comprehensive existing national citizen science programme which could be extended, following the GBH*i* model, to incorporate disease surveillance of other native species within garden habitats.

Reports could be taken on larger species (e.g. red fox (*Vulpes vulpes*)) but submissions for post mortem examination would be limited by practicable size for postal submission. Additional resources would be required to facilitate large carcass retrieval, such as those used by the CSIP, with significant cost implications.

Careful consideration would be required for disease investigation of wildlife species involving the general public, where significant zoonotic potential exists (e.g. European bat lyssavirus 2 in Daubenton bats (*Myotis daubentonii*) (Johnson et al., 2003) and bovine tuberculosis in European badgers (*Meles meles*) (Delahay et al., 2001)). However, a research group focusing broadly on garden wildlife health could provide information from related non-governmental organizations and forward enquiries directly to the appropriate government laboratories for investigation. Alternatively a collaborative approach could be established which would protect public health, prioritise screening for the relevant zoonotic pathogen(s), but ensure that comprehensive disease investigation could be practiced for these species to enable a broad understanding of the diseases that affect them to be gained rather than a restricted perspective based on a single pathogen.

iii. Disease Surveillance by bird ringers

A major limitation of this study is that it was restricted to bird species that visit garden habitats. Secondly the pathological investigations rely solely on post mortem examination; consequently, greatest focus is placed on infectious causes of mortality rather than on diseases that subclinically or incidentally affect birds. The licensed bird ringing community has regular contact with live wild bird species across habitat types in the UK. This scheme operates on an extensive scale with over 2000 licensed individuals catching in excess of 800,000 birds per annum (BTO 2010b). Data on each bird caught are recorded, for example the species, age and biometric measurements, using standardised EURING codes (Euring 2010). There is potential to amend this recording format to incorporate disease-based information, particularly on infections that result in clear external signs. Care would be needed to use syndromic codes, with relevant lists of differential diagnoses (e.g. chaffinch foot lesions due to cnemidocoptiasis or chaffinch papilloma virus infection); to avoid the impression that accurate diagnoses can be reached without confirmatory pathological investigation. Avian pox infection and ectoparasite burdens could also be recognised with confidence by the educated ringing community and are not typically considered to be primary causes of death. Ringers would be encouraged to take photographs of lesions and would be invited to contact the co-ordinating veterinary surgeon to investigate unusual observations. Collaboration with the ringing community offers the most cost-effective and logical next-step in developing wild bird surveillance across Great Britain.

LITERATURE CITED

Abulreesh HH, Coulder R, Scott GW (2007) Wild birds and human pathogens in the context of ringing and migration. Ringing and migration 23:193-200.

Adams CJ, Feldman SH, Sleeman JM (2005) Phylogenetic analysis of avian poxviruses among free-ranging birds of Virginia. Avian Diseases 49:601-605.

Adkesson MJ, Zdziarski JM, Little SE (2005) Atoxoplasmosis in tanagers. Journal of Zoo and Wildlife Medicine 36:265-272.

Alexa P, Salajka E, Hamrik J, Nejezchleb A (1995) Pathogenic strains of *Escherichia coli* in weaned piglets in the Czech Republic. Pig News Information 16:81-84.

Alley MR, Connolly JH, Fenwick SG, Mackereth GF, Leyland MJ, Rogers LE, Haycock M, Nicol C, Reed CEM (2002) An epidemic of salmonellosis caused by *Salmonella* Typhimurium DT160 in wild birds and humans in New Zealand. New Zealand Veterinary Journal 50:170-176.

Anderson ES (1968) Drug resistance in *Salmonella typhimurium* and its implications. British Medical Journal 3:333-339.

Anderson ES, Ward LR, de Saxe MJ, De Sa JDH (1977) Bacteriophage-typing designations of *Salmonella typhimurium*. Journal of Hygiene 78:297-300.

Anderson NL, Grahn RA, Van Hoosear K, Bondurant RH (2009) Studies of trichomonad protozoa in free ranging songbirds: Prevalence of *Trichomonas gallinae* in house finches (*Carpodacus mexicanus*) and corvids and a novel trichomonad in mockingbirds (*Mimus polyglottos*). Veterinary Parasitology 161:178-186.

Anon. (1999a) A better quality of life – a strategy for sustainable development for the United Kingdom. London, UK: Department for Environment, Transport and the Regions.

Anon. (1999b) Council Directive 1999/29/EC of 22 April 1999 on the undesirable substances and products in animal nutrition. Official Journal of the European Communities L115/32, 04/05/1999, pp. 1-15.

Anon. (2004a) The Feeding Stuffs, the Feeding Stuffs (Sampling and Analysis) and the Feeding Stuffs (Enforcement) (Amendment) (England) (No.2) Regulations 2004 Statutory Instrument No. 2688. HMSO, 2004, pp. 1-12.

Anon. (2004b) The Feeding Stuffs, the Feeding Stuffs (Sampling and Analysis) and the Feeding Stuffs (Enforcement) (Amendment) Regulations (Northern Ireland) 2004 Statutory Rule No. 443. HMSO, 2004, pp. 1-11.

Anon. (2004c) The Feeding Stuffs, the Feeding Stuffs (Sampling and Analysis) and the Feeding Stuffs (Enforcement) (Amendment) (Wales) (No.2) Regulations 2004 Welsh Statutory Instrument No. 3091 (W.265). HMSO, 2004, pp. 1-12.

Anon. (2004d) The Feeding Stuffs (Scotland) Amendment (No.2) Regulations 2004 Scottish Statutory Instrument 2004 No. 458, HMSO, 2004, pp. 1-10.

Anon. (2006) Council Recommendation 2006/576/EC of 17 August 2006 on the on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Official Journal of the European Communities, L 229/7, 23/08/2006, pp. 1-3.

Anon. (2007) Canker in wild birds. British Wildlife Rehabilitation Council Newsletter 45:5.

Anwar M (1966) *Isospora lacazei* (Labbe 1893) and *I. chloridis* sp. n. (Protozoa: Eimeriidae) from the English sparrow (*Passer domesticus*), Greenfinch (*Chloris chloris*) and Chaffinch (*Fringilla coelebs*). Journal of Protozoology 13:84-90.

AOAC (1995) AOAC Official Method 982.24 Aflatoxins B1 and M1 in liver, thin layer chromatography Method, Natural Toxins. In: Cunniff PA (Ed.) AOAC Official Methods of Analysis of AOAC International (16th Edition). Arlington, U.S.A.: AOAC, pp. 31-32.

Arcese P, Smith JNM (1988) Effects of population density and supplemental food on reproduction in song sparrows. Journal of Animal Ecology 57:119-136.

Baillie SR, Marchant JH, Crick HQP, Noble DG, Balmer DE, Beaven LP, Coombes RH, Downie IS, Freeman SN, Joys AC, Leech DI, Raven MJ, Robinson RA, Thewlis RM (2005) Breeding Birds in the wider countryside: their conservation status 2004. BTO Research Report No. 385. Thetford, UK: BTO, pp. 1-20.

Baird-Parker AC (1991) Foodborne salmonellosis. In: Waites WM, Arbuthnott JP (Eds.) Foodborne illness: a *Lancet* review. London, UK: Edward Arnold Ltd., pp. 53-61.

Baker JR (1977) The results of post-mortem examination of 132 wild birds. British Veterinary Journal 133:327-333.

Baker JR (1986) Trichomoniasis a major cause of vomiting in budgerigars. Veterinary Record 118:447-449.

Baker P, Molony SE, Stone E, Cuthill IC, Harris S (2008) Cats about town: is predation by free-ranging pet cats *Felis catus* likely to affect urban bird populations? Ibis 150:86-99.

Balcomb R (1986) Songbird carcasses disappear rapidly from agricultural fields. Auk 103:817-820.

Ball SJ, Brown MA, Daszak P, Pittilo RM (1998) Atoxoplasma (Apixomplexa: Eimeriorina: Atoxoplasmatidae) in the Greenfinch (*Carduelis chloris*). Journal of Parasitology 84:813-817.

Balmer G, Coiffait L, Clark J, Robinson R (2008) Bird ringing: a concise guide. Thetford, UK: BTO, pp. 1-75.

Barrow PA, Bumstead N, Marson K, Lovell MA, Wigley P (2004) Faecal shedding and intestinal colonization of *Salmonella* enterica in in-bred chickens: the effect of host-genetic background. Epidemiology and Infection 132:117-26.

Bauman KM, Hawley DM (2010) Sickness behaviour acting as an evolutionary trap? Male house finches preferentially feed near diseased conspecifics. Biology Letters doi: 10.1098/rsbl.2010.0020

Bellrose FC (1945) Aspergillosis in wood ducks. Journal of Wildlife Management 9:325-326.

Benchimol M (2004) Trichomonads under microscopy. Microscopy and Microanalysis 10:528-550.

Benskin CMW, Wilson K, Jones K, Hartley IR (2009) Bacterial pathogens in wild birds: a review of the frequency and effects of infection. Biological Reviews 84:349-373.

Bintvihok A, Thiengnin S, Doi K, Kumagai S (2002) Residues of aflatoxins in the liver, muscle and eggs of domestic fowls. Journal of Veterinary Medical Science 64:1037-1039.

Biodiversity Action Reporting System (2008) Action Plan details – The London Biodiversity Partnership. Available: http://www.ukbap-reporting.org.uk/plans/ [Accessed January 2010].

(BSA) BirdCare Standards Association (2009) Standards. Available:.http://www.birdcare.org.uk/standards.htm [Accessed September 2009].

Blackmore DK, Keymer IF (1969) Cutaneous diseases of wild birds in Britain. British Birds 62:316-331.

Blanco M, Blanco J, Blanco JE, Ramos J (1993) Distribution and characterization of faecal necrotoxigenic *Escherichia coli* $CNF1^+$ and $CNF2^+$ isolated from healthy cows and calves. Research in Microbiology 59:183-192.

Bland RL, Tully J, Greenwood JJD (2004) Birds breeding in British gardens: an underestimated population? Bird Study 51:96–106.

Blount WP (1961) Turkey "X" disease. Turkeys 9:52-77.

Bondurant RH, Honigberg BM (1994) Trichomonads of veterinary importance. In: Kreier JP (Ed.) Parasitic Protozoa. New York, U.S.A.: Academic Press, pp. 111-206.

Boyd EM (1951) A survey of parasitism of the starling *Sturnus vulgaris* in North America. Journal of Parasitology 37:56-84.

Bradley CA, Altizer S (2007) Urbanization and the ecology of wildlife diseases. Trends 22:95-102.

(BTO) British Trust for Ornithology (2006) Headline indicators for sustainable development, Populations of wild birds in the UK, 1970-2005. Available: http://www.bto.org/research/indicators/uk_indicators.htm [Accessed January 2010].

BTO (2008) Garden BirdWatch – Garden Mammals, Tracking Mammals Partnership. Available: http://www.bto.org/gbw/mammals/index.htm [Accessed January 2010].

BTO (2009) Garden BirdWatch – Reptiles and Amphibians in Gardens 2009 Survey. Available: http://www.bto.org/herps/2009_survey/index.htm [Accessed January 2010].

BTO (2010) British Trust for Ornithology Garden BirdWatch Results and Maps. Available: http://www.bto.org/gbw [Accessed March 2010].

BTO (2010b) Bird Ringing in Britain and Ireland: The Ringing Scheme. Available: http://www.bto.org/ringing [Accessed February 2010].

Brittingham MC, Temple SA (1986) A survey of avian mortality at winter feeders. Wildlife Society Bulletin 14:445-450.

Brittingham MC, Temple SA (1988) A survey of the prevalence of selected bacteria in wild birds. Journal of Wildlife Diseases 24:288-307.

Brittingham MC, Temple SA (1992a) Use of winter bird feeders by black-capped chickadees. Journal of Wildlife Management 56:103-110.

Brittingham MC, Temple SA (1992b) Does winter bird feeding promote dependency? Journal of Field Ornithology 63:190-194.

Brown JD, Stallknecht DE, Swayne DE (2008) Experimental infection of swans and geese with highly pathogenic avian influenza virus (H5N1) of Asian lineage. Emerging Infectous Disease 14:136-142.

Brown MA, Ball SJ, Holman D (2001) The periodicity of isosporan oocyst discharge in the greenfinch (*Carduelis chloris*). Journal of Natural History 35:945-948.

Buenestado F, Gortazar C, Millan J, Hofle U, Villafuerte R (2004) Descriptive study of an avian pox outbreak in wild red-legged partridges (*Alectoris rufa*) in Spain. Epidemiology and Infection 132:369-374.

Bunbury N, Bell D, Jones C, Greenwood A, Hunter P (2005) Comparison of the InPouch TF culture system and wet-mount microscopy for diagnosis of *Trichomonas gallinae* infections in the Pink Pigeon *Columba mayeri.* Journal of Clinical Microbiology 43:1005-1006.

Bunbury N (2006) Parasitic disease in the endangered Mauritian Pink Pigeon *Columba mayeri.* PhD thesis. School of Biological Sciences, University of East Anglia, Norfolk, UK

Bunbury N, Jones CG, Greenwood AG, Bell DJ (2007) *Trichomonas gallinae* in Mauritian columbids: implications for an endangered endemic. Journal of Wildlife Diseases 43:399-407.

Bunbury N, Jones CG, Greenwood AG, Bell DJ (2008) Epidemiology and conservation implications of *Trichomonas gallinae* infection in the endangered Mauritian pink pigeon. Biological Conservation 141:153-161.

Cacciuttolo E, Rossi G, Nardoni S, Legrottagllie R, Mani P (2009) Anatomopathological aspects of avian aspergillosis. Veterinary Research Communication [Epub ahead of print].

Callender GR, Simmons JS (1937) Trichomoniasis (*T. columbae*) in the Java sparrow, Tovi parakeet and Verraux's dove. American Journal of Tropical Medicine 17:579-585.

Cannon AR, Chamberlain DE, Toms MP, Hatchwell BJ, Gaston KJ (2005) Trends in the use of private gardens by wild birds in Great Britain, 1995-2002. Journal of Applied Ecology 42:659-671.

(CEN) European Committee for Standardization (1999) Food Analysis – Biotoxins - Criteria of analytical methods of mycotoxins. Report Reference Number CR 13505. Brussels, Belgium: CEN.

Cepicka I, Kutisova K, Tachezy J, Kulda J, Flegr J (2005) Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism. Veterinary Parasitology 128:11-21.

Chalmers GA (1992) Trichomoniasis in finches. Cross-Canada Disease Report Canadian Veterinary Journal 33:616-617.

Chamberlain DE, Vickery JA, Glue DE, Robinson RA, Conway GJ, Woodburn RJW, Cannon AR (2005) Annual and seasonal trends in the use of garden feeders by birds in winter. Ibis 147:563-575.

Chan D, MacDonald SJ, Boughtflower V, Brereton P (2004) Simultaneous determination of aflatoxins and ochratoxin A in food using a fully automated immunoaffinity column clean-up and liquid chromatography-fluorescence detection. Journal of Chromatography A 1059:13-16.

Chang CF, Doerr JA, Hamilton PB (1981) Experimental ochratoxicosis in turkey poults. Poultry Science 60:114-119.

Chastel C, Monnat JY, le Lay G, Beaucournu JC (1981) Fatal neurological syndrome in a collared dove (*Streptopelia decaoacto*) and new host of *Ixodes Pari* (=*Ixodes frontalis*). Annales de Parasitologie Humaine et Comparee 56:349-351.

Chowdhury K (1991) One step 'Miniprep' method for the isolation of plasmid DNA. Nucleic Acids Research 19:2792.

Chvala S, T. Bakonyi C, Bukovsky T, Meistera K, Bruggerd F, Rubel F, Nowotny N, Weissenböck H (2007) Monitoring of Usutu virus activity and spread by using dead bird surveillance in Austria, 2003–2005. Veterinary Microbiology 122:237-245.

Clement P, Harris A, Davis J (1993) Finches & sparrows an identification guide. London, UK: Christopher Helm, A&C Black, pp. 1-500.

Cole RA (1999a) Trichomoniasis. In: Friend M, Franson JC (Eds.) Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds. U.S.A.: Biological Resources Division Information and Technology Report 1999-2001, pp. 201-206.

Cole RA (1999b) Acanthocephaliasis. In: Friend M, Franson JC (Eds.) Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds. U.S.A.: Biological Resources Division Information and Technology Report 1999-2001, pp. 241-243.

Cole RA (1999c) Tracheal worms. In: Friend M, Franson JC (Eds.) Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds. U.S.A.: Biological Resources Division Information and Technology Report 1999-2001, pp. 229-232.

Collins P, McDiarmid A, Thomas LH, Matthews PR (1985) Comparison of the pathogenicity of *Mycobacterium paratuberculosis* and *Mycobacterium* spp isolates from the wood pigeon (*Columba palumbus*-L). Journal of Comparative Pathology 95:591-597.

Connolly JH, Alley MR, Dutton GJ, Rogers LE (2006) Infectivity and persistence of an outbreak strain of *Salmonella enterica* serotype Typhimurium DT160 for house sparrows (*Passer domesticus*) in New Zealand. New Zealand Veterinary Journal 54:329-332.

Converse KA (2007) Aspergillosis. In: Thomas NJ, Hunter DB, Atkinson CT (Eds.) Infectious Diseases of Wild Birds. Oxford, UK: Wiley-Blackwell Publishing, pp. 239-269.

Cooper CB, Dickinson J, Phillips T, Bonney R (2007) Citizen science as a tool for conservation in residential ecosystems. Ecology and Society 12:11.

Cooper JE, Gschmeissner S, Greenwood AG (1989) Atoxoplasma in greenfinches (*Carduelis chloris*) as a possible cause of 'going light'. Veterinary Record 124:343-344.

Cornish TE, Nettles VF (1999) Aflatoxicosis in Louisiana geese. Southeastern Co-operative Wildlife Diseases Study Briefs 15:1-2.

Cousquer G (2003) Trichomoniasis in wild birds presented to a southwest wildlife hospital (1998-2002). Proceedings of the British Veterinary Zoological Society Meeting on Infectious diseases of Exotics, Edinburgh, UK, November 2003, pp. 81-88.

Couvillion CE, Jackson JR, Ingram RP, Bennett LW, McCoy CP (1991) Potential natural exposure of Mississippi Sandhill cranes to aflatoxin B_1. Journal of Wildlife Diseases 27:650-656.

Cowie RJ, Hinsley SA (1988) Feeding ecology of great tits (*Parus major*) and blue tits (*Parus caeruleus*) breeding in suburban gardens. Journal of Animal Ecology 57:611-626.

Crawley MJ (2002) Statistical computing – an introduction to data analysis using S-Plus. Chichester, UK: Wiley, pp. 1-761.

Creekmore LH (1999) Mycotoxins. In: Friend M, Franson JC (Eds.) Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds. U.S.A.: Biological Resources Division Information and Technology Report 1999-2001, pp. 267-270.

Crick HQP, Robinson RA, Siriwardena GM (2002) Causes of the population declines: summary and recommendations. In: Crick HQP, Robinson RA, Appleton GF, Clark NA, Rickard AD (Eds.) Investigation into the causes of the declines of Starlings and House Sparrows in Great Britain. London, UK: BTO Research Report No 290, Department of Environment, Food and Rural Affairs, pp. 265-292.

Cunningham AA, Langton TE, Bennett PM, Lewin JF, Drury SE, Gough RE, Macgregor SK (1996) Pathological and microbiological findings from incidents of unusual mortality of the common frog *(Rana temporaria)*. Philosophical Transactions of the Royal Society of London B: Biological Sciences 351:1539-57.

Cunningham AA, Garner TWJ, Aguilar-Sanchez V, Banks B, Foster J, Sainsbury AW, Perkins M, Walker SF, Hyatt AD, Fisher M (2005) Emergence of amphibian chytridiomycosis in Britain. Veterinary Record 157:386.

Cunningham AA, Hyatt AD, Russell P, Bennett PM (2007) Emerging epidemic diseases of frogs in Britain are dependent on the source of ranavirus agent and the route of exposure. Epidemiology and Infection 135:1200-1212.

Dalessi S, Hoop R, Engels M (2007) The 2005/2006 avian influenza monitoring of wild birds and commercial poultry in Switzerland. Avian Diseases 51:355-358.

Daniels MJ, Hutchings MR, Greig A (2003) The risk of disease transmission to livestock posed by contamination of farm stored feed by wildlife excreta. Epidemiology and Infection 130:561-568.

Daoust PY, Busby DG, Ferns L, Goltz J, McBurney S, Poppe C, Whitney H (2000) Salmonellosis in songbirds in the Canadian Atlantic provinces during winter-summer 1997-98. Canadian Veterinary Journal 41:54-59.

Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife-threats to biodiversity and human health. Science 287:443-449.

Daszak P, Cunnngham AA, Hyatt AD (2001) Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica 78:103-116.

Daszak P, Tabor GM, Kilpatrick AM, Epstein J, Plowright R (2004) Conservation medicine and a new agenda for emerging diseases. Annals of the New York Academy of Sciences 1026:1-11.

Davidson WR, Kellogg FE, Doster GL (1980) An epornitic of avian pox in wild bobwhite quail. Journal of Wildlife Diseases 16:293-298.

Davies ZG, Fuller RA, Loram A, Irvine KN, Sims V, Gaston KJ (2009). A national scale inventory of resource provision for biodiversity within domestic gardens. Biological Conservation 142:761-771.

Deaville R, Jepson PD (2009) UK Cetacean Strandings Investigation Programme Annual Report for the period 1st January – 31st December 2008. pp. 1-56. Available: http://ukstrandings.org/csip-reports/. [Accessed February 2010].

Delahay RJ, Cheeseman CL, Clifton-Hadley RS (2001) Wildlife disease reservoirs: the epidemiology of *Mycobacterium bovis* infection in the European badger (*Meles meles*) and other British mammals. Tuberculosis 81:43-50.

DeGraaf RM, Payne BR (1975) Economic values of non-game birds and some urban wildlife resource needs. Transactions of the North American Wildlife and Natural Resources Conference 40:281-287.

Dein FJ (2001) Disease emergence in birds: challenges for the twenty-first century. Auk 118:290-303.

Delgado-Viscogliosi P, Viscogliosi E, Gerbod D, Kulda J, Sogin ML, Edgcomb VP (2000) Molecular phylogeny of Parabasalids based on small subunit rRNA sequences, with emphasis on the Trichomonadinae subfamily. The Journal of Eukaryote Microbiology 47:70-75.

(Defra) Department for Environment, Food and Rural Affairs (2009) Natural resource protection and enhancing the environment: Bird populations. Available: http://www.defra.gov.uk/sustainable/government/progress/national/20.htm [Accessed January 2010].

Devegowda G, Murthy TNK (2005) Mycotoxins: their effects in poultry and some practical solutions. In: Diaz D (Ed.) The Mycotoxin Blue Book. Nottingham, UK: Nottingham University Press, pp. 1-350.

Dhondt AA, Tessaglia DL, Slothower RL (1998) Epidemic mycoplasmal conjunctivitis in house finches from eastern North America. Journal of Wildlife Diseases 34:265-280.

Doby JM, Bigaignon G (1997) Ixodid ticks parasitizing birds and their pathogenic role. Revue-de-Medicine-Veterinaire 148:853-860.

Duff JP (2002) Wildlife diseases in the UK: Annual report to the Department of Environment, Food and Rural Affairs and the Office International Épizooties, pp. 1-32.

Duff JP, Higgins R, Farrelly S (2007) Enteric adenovirus infection in a red squirrel (*Sciurus vulgaris*).Veterinary Record 160:384.

Dumonceaux G, Harrison GJ (1994) Toxins. In: Ritchie BW, Harrison GJ, Harrison LR (Eds.) Avian Medicine: Principles and Application.Lake Worth, U.S.A.:Wingers Publishing Inc., pp. 1030-1052.

Dwivedi P, Burns RB (1984a) Pathology of ochratoxicosis A in young broiler chicks. Research in Veterinary Science 36:92-103.

Dwivedi P, Burns RB (1984b) Effect of ochratoxin A on immunoglobulins in broiler chicks. Research in Veterinary Science 36:117-121.

EC (2006) Summary Minutes of the meeting of the Standing Committee on the Food Chain and Animal Health, Animal Health Section. Available: http://ec.europa.eu/food/committees/regulatory/scfcah/animalnutrition/summary38_en.pdf. [Accessed February 2008].

Edgcomb VP, Viscogliosi E, Simpson AGB, Delgado-Viscogliosis P, Roger AJ, Sogin ML (1998) New insights into the phylogeny of trichomonads inferred from small subunit rRNA sequences. Protist 149:359-366.

Edwards GR (1955) Excrescenses about the eyes and on the legs and feet of dunnocks. British Birds 48:186-187.

Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, Mostashari F, McClean R (2001) Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. Emerging Infectious Diseases 7:615-620.

Eley C (1991) Status signalling in the western greenfinch (*Carduelis chloris*). PhD thesis. University of Sussex, Brighton, UK.

Erwin KG, Kloss C, Lyles J, Felderhoff J, Fedynich AM, Henke SE, Roberson JA (2000) Survival of *Trichomonas gallinae* in white-winged dove carcasses. Journal of Wildlife Diseases 36:551-554.

Euden PR (1990) Salmonella isolates from wild animals in Cornwall. British Veterinary Journal 146:228-232.

(Euring) The European Union for Bird Ringing (2010). Euring. Available: http://www.euring.org/index.html [Accessed January 2010].

Everest DJ, Dastjerdi A, Gurrala R, Banks M, Meredith AL, Milne EM, Sainsbury AW (2009) Rotavirus in red squirrels from Scotland. Veterinary Record 165:450.

Ewing WH (1986) Edwards & Ewing identification of *Enterobacteriaceae*, 4th edition. New York, U.S.A.: Elsevier Science Publishing, pp. 1-536.

Faddoul GP, Fellows GW, Baird J (1966) A survey on the incidence of Salmonellae in wild birds. Avian Diseases 10:89-94.

Feare C (1984) The starling. Oxford, U.K: Oxford University Press, pp. 1-315.

Felicia WU (2004) Mycotoxin risk assessment for the purpose of setting international regulatory standards. Environmental Science and Technology 38:4049-4055.

Felleisen RSJ (1997) Comparative sequence analysis of 5.8S rRNA genes and internal transcribed spacer (ITS) regions of trichomonadid protozoa. Parasitology 115:111-119.

Felleisen RSJ (1998) Comparative genetic analysis of tritrichomonadid protozoa by the random amplified polymorphic DNA technique. Parasitology Research 84:153-156.

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39:783-791.

Fischer JR, Jain AV, Shipes DA, Osborne JS (1995) Aflatoxin contamination of corn used as bait for deer in the Southeastern United States. Journal of Wildlife Diseases 31:570-572.

Food Standards Agency (2003) Aflatoxins in wild bird foods. Available: http://www.food.gov.uk/consultations/ukwideconsults/2003/aflatoxinsinwildbirdfoods [Accessed June 2009].

Forrester DJ, Foster GW (2008) Trichomonosis. In: Atkinson CT, Thomas NJ, Hunter DB (Eds.) Parasitic diseases of wild birds. Iowa, U.S.A.: Wiley-Blackwell, pp. 120-153.

Forzán MJ (2009) Trichomoniasis in wild finches. Canadian Veterinary Journal 50:561.

Forzán MJ, Vanderstichel R, Melekhovets YF, McBurney S. (2010) Trichomoniasis in finches from the Canadian maritime provinces, an emerging disease. Canadian Veterinary Journal 51:391-396.

Foster G, Ross HM, Pennycott TW, Hopkins GF, McLaren IM (1998) Isolation of *Escherichia coli* O86:K61 producing cyto-lethal distending toxin from wild birds of the finch family. Letters in Applied Microbiology 26:395-398.

Foster G, Malnick H, Lawson PA, Kirkwood J, Macgregor SK, Collins MD (2005) *Suttonella ornithocola* sp. nov., from birds of the tit families, and emended description of the genus *Suttonella*. International Journal of Systematic and Evolutionary Microbiology 55:2269-2272.

Francki RIB, Fauquet CM, Knudson DL, Brown F (1991) Classification and nomenclature of viruses. Fifth Report on the International Committee on Taxonomy of Viruses. Archives of Virology, Supplement. New York, U.S.A.: Springer-Verlag, pp. 1-450.

Freshwater A (2008) Why your housecat's trite little bite could cause you quite a fright: a study of domestic felines on the occurrence and antibiotic susceptibility of *Pasteurella multocida*. Zoonoses and Public Health 55:507-513.

Friend M (1999a) Salmonellosis. In: Friend M, Franson JC (Eds.) Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds. Madison, U.S.A.: Biological Resources Division Information and Technology Report 1999-2001, pp. 201-206.

Friend M (1999b) Mycobacteriosis. In: Friend M, Franson JC (Eds.) Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds. U.S.A.: Biological Resources Division Information and Technology Report 1999-2001, pp. 93-98.

Friend M (1999c) Aspergillosis. In: Friend M, Franson JC (Eds.) Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds. U.S.A.: Biological Resources Division Information and Technology Report 1999-2001, pp. 130-133.

Fuller RA, Warren PH, Armsworth PR, Barbosa O, Gaston KJ (2008) Garden bird feeding predicts the structure of urban avian assemblages. Diversity and Distributions 14:131–137.

Galvano F, Ritieni A, Piva G, Pietri A (2005) Mycotoxins in the human food chain. In: Diaz D (Ed.) The Mycotoxin Blue Book. Nottingham, UK: Nottingham University Press, pp. 187-224.

(GBH*i*) Garden Bird Health *initiative* (2005) UFAW Programmes: The Garden Bird Health *initiative*. Available http://www.ufaw.org.uk/gbhi.php [Accessed June 2009].

Gaspar da Silva D, Barton E, Bunbury N, Lunness P, Bell DJ, Tyler KM (2007) Molecular identity and heterogeneity of Trichomonad parasites in a closed avian population. Infection Genetics and Evolution 7:433-440.

Gaston KJ, Fuller RA, Loram A, MacDonald C, Power S, Dempsey N (2007) Urban domestic gardens (XI): variation in urban wildlife gardening in the United Kingdom. Biodiversity and Conservation 16:3227–3238.

Gauthier-Clerc M, Lebarbenchon C, Thomas F (2007) Recent expansion of highly pathogenic avian influenza H5N1: a critical review. Ibis 149:202-214.

Gerbod D, Sanders E, Moriya S, Nöel C, Takasu H, Fast NM, Delagado-Viscogliosi P, Ohkuma M, Kudo T, Capron M, Palmer JD, Keeling PJ, Viscogliosis E (2004) Molecular phyogenies of Parabasalia inferred from four protein genes and comparison with rRNA trees. Molecular Phylogenetics and Evolution 31:572-580.

Gerhold RW, Yabsley MJ, Smith AJ, Ostergaard E, Mannan W, Cann JD, Fischer JR (2008) Molecular characterization of the *Trichomonas gallinae* morphologic complex in the United States. Journal of Parasitology 94:1335-1341.

Gibbons DW, Reid JB, Chapman RA (1993) The New Atlas of Breeding Birds in Britain and Ireland: 1988–1991. London, UK: T. & A.D. Poyser, pp. 1-520.

Gilbert M, Xiao X, Domenech J, Lubroth J, Martin V, Slingenbergh J (2006) Anatidae migration in the western Palearctic and spread of highly pathogenic avian influenza H5NI virus. Emerging Infectious Diseases 12:1650-1656.

Goddard MA, Dougill AJ, Benton TG (2009) Scaling up from gardens: biodiversity conservation in urban environments. Trends in Ecology and Evolution 25:90-98.

Gordon MA (2008) Salmonella infections in immunocompromised adults. Journal of Infection 66:413-422.

Gould G (2007) An investigation into seasonal tick-related syndrome in collared doves (*Streptopelia decaocto*). Royal Veterinary College Final Year Research Project. Royal Veterinary College, London, UK, pp. 1-42.

Gourama H, Bullerman LB (1995) *Aspergillus flavus* and *Aspergillus parasiticus*: Aflatoxigenic fungi of concern in foods and feeds: a review. Journal of Food Protection 58:1395-1404.

Grant D, Todd PA, Pennycott T (2007) Monitoring wild greenfinch (*Carduelis chloris*) for *Salmonella enterica typhimurium*. Ecological Research 22:571-574.

Great Backyard Bird Count (2010) Great Backyard Bird Count. Available: http://www.birdsource.org/gbbc/ [Accessed January 2010].

Gregory JF, Goldstein SL, Edds GT (1983) Metabolite distribution and rate of residue clearance in turkeys fed a diet containing aflatoxin B_1. Food and Chemical Toxicology 21:463-467.

Gregory RD, Baille SR (1998) Large-scale habitat use of some declining British birds. Journal of Applied Ecology 35:785-799.

Grubb TC, Cimprich DA (1990) Supplementary food improves the nutritional condition of wintering woodland birds: evidence from ptilochronology. Ornis Scandinavica 21:277-281.

Gruber A, Grabensteiner E, Kolodziejek J, Nowotny N, Loupal G (2007) Poxvirus in a great tit (*Parus major*). Avian Diseases 51:623-625.

Gruwell JA, Fogarty CL, Bennett SG, Challet GL, Vanderpool KS, Jozan M, Webb JP (2000) Role of peridomestic birds in the transmission of St. Louis encephalitis virus in southern California. Journal of Wildlife Diseases 36:13-34.

Gunderson J, Hinkle G, Leipe D, Morrison HG, Stickel SK, Odelson DA, Breznak JA, Nerad TA, Muller M, Sogin ML (1995) Phylogeny of trichomonads inferred from small-subunit rRNA sequences. The Journal of Eukaryote Microbiology 42:411-415.

Gyuranecz M, Károly E, Höfle U, Dorrestein GM, Szabolcs S, Blanco HM, Ádám D (2007) The phylogenetic analysis of avian pox isolates from Hungarian, Spanish and Dutch captive and wild birds. European Wildlife Disease Association Student Workshop, Greece, April 2007, p. 6.

Hall AJ, Saito EK (2008) Avian wildlife mortality events due to salmonellosis in the United States, 1985-2004. Journal of Wildlife Diseases. 44:585-593.

Hamilton PB, Huff WE, Harris JR, Wyatt RD (1982) Natural occurrences of ochratoxicosis in poultry. Poultry Science 61:1832-1841.

Handeland K, Nesse LL, Lillehaug A, Vikøren T, Djønne B, Bergsjø B (2008) Natural and experimental *Salmonella* Typhimurium infections in foxes. Veterinary Microbiology 132:129-134.

Hansen WR (1999) Avian pox. In: Friend M, Franson JC (Eds.) Field Manual of Wildlife Diseases General Field Procedures and Diseases of Birds. U.S.A.: Biological Resources Division Information and Technology Report 1999-2001, pp. 163-170.

Hars J, Ruette S, Benmergui M, Fouque C, Fournier JY, Legouge A, Cherbonnel M, Daniel B, Dupuy C, Jestin V (2008) The epidemiology of the highly pathogenic H5N1 avian influenza in mute swan (*Cygnus olor*) and other Anatidae in the Dombes region (France), 2006. Journal of Wildlife Diseases 44:811-823.

Hartup BJ, Kollias GV (1999) Field investigation of *Mycoplasma gallisepticum* infections in house finch (*Carpodacus mexicanus*) eggs and nestlings. Avian Diseases 43:572-576.

Haugen AO (1952) Trichomoniasis in Alabama mourning doves. Journal of Wildlife Management 16:164-169.

Haugen AO, Keeler J (1952) Mortality of mourning doves from trichomoniasis in Alabama during 1951. Transactions of the North American Wildlife Conference 17:141-151.

Hauser E, Huhn S, Junker E, Jaber M, Schroeter A, Helmuth R, Rabsche W, Winterhoff N, Malomy B (2009) Characterisation of a phenotypic monophasic variant belonging to *Salmonella enterica* subsp. enterica serovar Typhimurium from wild birds and its possible transmission to cats and humans. Berl Munch Tierarztl Wochenschr 122:169-177.

(HPA) Health Protection Agency (2005) Salmonella in humans. Available: http://www.hpa.org.uk/infections/topics_az/salmonella [Accessed June 2005].

HPA (2010) Communicable Disease Report Weekly Available: http://www.hpa.org.uk/cdr/default.htm [Accessed January 2010].

(HSE) Health and Safety Executive (2010) Wildlife Incident Investigation Scheme Available: http://www.pesticides.gov.uk/environment. [Accessed February 2010].

Heir E, Lindstedt BA, Nygard I, Vardund T, Hasseltvedt V, Kapperud G (2002) Molecular epidemiology of Salmonella Typhimurium isolates from human sporadic and outbreak cases. Epidemiology and Infection 128:373-382.

Henke SE, Moore DL, Fedynich AM, Laurenz JC (2004) Assessment of aflatoxin toxicity in granivorous avian species. Proceedings of the 2004 American Association of Zoo Veterinarians, American Association of Wildlife Veterinarian and Wildlife Disease Association Joint Conference, San Diego, U.S.A. August 2004, pp. 575-576.

Hernandez J, Bonnedahl J, Waldenström J, Palmgren H, Olsen B (2003) Salmonella in birds migrating through Sweden. Emerging Infectious Diseases 9:753-755.

Higgins RJ (1980) Caecal trichomoniasis of game birds in North Yorkshire. Veterinary Record 107:228.

Higgs S, Snow K, Gould EA (2004) The potential for West Nile virus to establish outside of its natural range: a consideration of potential mosquito vectors in the United Kingdom. Transactions of the Royal Society of Tropical Medicine and Hygiene 98:82-87.

Hochachka WM, Dhondt AA (2000) Density-dependent decline of host abundance resulting from a new infectious disease. Proceedings of the National Academy of Science USA 97:5303-5306.

Hof A (2009) A study of the current status of the hedgehog (*Erinaceus europaeus*) and its decline in Great Britain. PhD thesis. Royal Holloway, University of London, London, UK

Höfle U, Blanco J, Palma L, Melo P (2000) Trichomoniasis in Bonelli's eagle (*Hieraaetus fasciatus*) nestlings in south-west Portugal. In: Lumej JT, Remple JD, Redig PT, Lierz M, Cooper JE (Eds.) Raptor Biomedicine III. Lake Worth, Florida, U.S.A.: Zoological Education, Network Inc., pp. 4-52.

Höfle U, Gortazar C, Irtiz JA, Knispel B, Kaleta EF (2004) Outbreak of trichomoniasis in a woodpigeon (*Columba palumbus*) wintering roost. European Journal of Wildlife Research 50:73-77.

Holley RA, Arrus KM, Ominski KH, Tenuta M, Blank G (2006) Salmonella survival in manure-treated soils during simulated seasonal temperature exposure. Journal of Environmental Quality 35:1170-1180.

Holmes P, Duff P (2005) Ingluvitis and oesophagitis in wild finches. Veterinary Record 157:455.

Holt G, Krogsrud J (1973) Pox in wild birds. Acta Veterinaria Scandinavica 14:201-203.

Hopkins KL, Threlfall EJ (2004) Frequency and polymorphism of SopeE in isolates of Salmonella enterica belonging to the ten most prevalent serotypes in England and Wales. Journal of Medical Microbiology 53:539-543.

Horak P, Saks L, Karu U, OTS I, Surai PF, MacGraw KJ (2004) How coccidian parasites affect health and appearance of greenfinches. Journal of Animal Ecology 73:935-947.

Hosseini PR, Dhondt AA, Dobson AP (2006) Spatial spread of an emerging infectious disease: conjunctivitis in house finches. Ecology 87:3037-3046.

Hudson CB, Tudor DC (1957) *Salmonella typhimurium* infection in feral birds. Cornell Veterinarian 47:394-395.

Hudson PJ, Dobson AP (1989) Population biology of *Trichostrongylus tenuis*, a parasite of economic importance for red grouse management. Parasitology Today 5:283-291.

Huff WE, Wyatt RD, Tucker TL, Hamilton PB (1974) Ochratoxicosis in the broiler chicken. Poultry Science 53:1585-1591.

Huff WE, Doerr JA (1981) Synergism between aflatoxin and ochratoxin A in broiler chickens. Poultry Science 60:550-555.

Huff WE, Ruff MD, Chute MB (1992) Characterisation of the toxicity of the mycotoxins aflatoxin, ochratoxin, and T-2 Toxin in game birds. II. Ringneck Pheasant. Avian Diseases 36:30-33.

Hughes LA, Shopland S, Wigley P, Bradon H, Leatherbarrow AH, Williams NJ, Bennett M, de Pinna E, Lawson B, Cunningham AA, Chantrey J (2008) Characterisation of *Salmonella* enterica serotype Typhimurium isolates from wild birds in northern England from 2005 - 2006. BMC Veterinary Research 29:4.

Hurvell B, Jevring J (1974) *Salmonella typhi-murium* in small passerines in Sweden. Nordisk Veterinærmedicin 26:392-399.

ICMSF (International Commission on Microbiological Specifications for Foods) (1996) Microorganisms in foods 5. (Food Safety) Characteristics of Microbial Pathogens. London, UK: Blackie Academic and Professional, pp. 1-513.

Immerseel FV, Pasmans F, De Buck J, Rychlik I, Hradecka H, Collard JM, Wildemauwe C, Heyndrickx M, Ducatelle R, Haesebrouck F (2004) Cats as a risk for transmission of antimicrobial drug-resistant Salmonella. Emerging Infectious Diseases 10:2169-2174.

Jenkins IM (2007) Prevalence of *Trichomonas gallinae* in feral pigeon (*Columba livia*) in London and rescue centres. Royal Veterinary College Undergraduate Research Project. Royal Veterinary College, London, UK

Jennings AR (1954) Diseases in wild birds. Journal of Comparative Pathology 64:356-359.

Jennings AR (1961) An analysis of 1000 deaths in wild birds. Bird Study 8:25-31.

Investigating potential associations between chronic exposure to polychlorinated biphenyls and infectious disease mortality in harbour porpoises from England and Wales.
Jepson PD, Bennet PM, Deaville R, Allchin CR, Baker JR, Law RJ (2005) Relationships between polychlorinated biphenyls and health status in harbor porpoises (Phocoeana phoceana) stranded in the United Kingdom. Toxicological and Environmental Chemistry 24:238-248.

(JNCC) Joint Nature Conservation Committee (2008) UK Biodiversity Action Plan. Available: http://www.ukbap.org.uk/ [Accessed January 2010].

Johnson N, Selden D, Parsons G, Healy D, Brookes SM, McElhinney MM, Hutson AM, Fooks AR (2003) Isolation of a European bat lyssavirus type 2 from a Daubenton's bat in the United Kingdom. Veterinary Record 152:383-387.

Jones PW, Twigg GI (1976) Salmonellosis in wild mammals. Journal of Hygiene 77:51-54.

Karstad L (1971) Pox. In: Davis JW, Anderson RC, Karstad L, Trainers DO (Eds.) Infectious and Parasitic Diseases of Wild Birds. Iowa, U.S.A.: Iowa State University Press, pp. 34-41.

Kapperud G, Stenwig H, Lassen J (1998) Epidemiology of *Salmonella typhimurium* O:4-12 infection in Norway: evidence of transmission from an avian wildlife reservoir. American Journal of Epidemiology 147:774-782.

Keawcharoen J, van Riel D, van Amerongen G, Bestebroer T, Beyer WE, van Lavieren R, Osterhaus AD, Fouchier RA, Kuiken T (2008) Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1). Emerging Infectious Diseases 14:600-607.

Keymer IF (1958) A survey and review of the causes of mortality in British birds and the significance of wild birds as disseminators of disease. Veterinary Record 70:713-720.

Keymer IF, Blackmore DK (1964) Diseases of the skin and soft parts of wild birds. British Birds 57:175-179.

Keymer IF (1997) Mycobacterium infections of birds. Veterinary Record 140:292.

Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP, Daszak P (2006) Predicting the global spread of H5N1 avian influenza. Proceedings of the National Academy of Sciences of the United States of America 103:19368-19373.

Kirkwood JK, Holmes JP, Macgregor SK (1995) Garden bird mortalities. Veterinary Record 136:372.

Kirkwood JK, Macgregor SK (1997) Infectious disease of garden birds: minimising the risks. Wheathampstead, UK: Universities Federation for Animal Welfare, pp. 1-16.

Kirkwood JK (1998) Population density and infectious disease at bird tables. Veterinary Record 142:468.

Kirkwood JK, Macgregor SK (1998) Salmonellosis in provisioned greenfinches (*Carduelis chloris*) and other garden birds. Proceedings of the Scientific Meeting of the European Association of Zoo and Wildlife Veterinarians, Chester, UK, May 2008, pp. 455-458.

Kirkwood JK, Macgregor SK, Malnick H, Foster G (2006) Unusual mortality incidents in tit species (family Paridae) associated with the novel bacterium *Suttonella ornithocola*. Veterinary Record 158:203-205.

Kirmse P (1967) Pox in wild birds, an annotated bibliography. Journal of Wildlife Diseases 49:1-20.

Kleina P, Bettim-Bandinelli J, Bonatto SL, Benchimol M, Bogo MR (2004) Molecular phylogeny of Trichomonadidae family inferred from ITS-1, 5.8S rRNA and ITS-2 sequences. International Journal of Parasitology 34:963-970.

Knispel BHM (2005) Morphologische und molekularbiologische Untersuchungen (PCR und REA der 5,8S rRNA-region) an *Trichomonas gallinae* und *Tetratrichomonas gallinarum* verschiedener Vogelarten. PhD thesis. Justus Liebig Universitat, Giessen, Germany.

Krone O, Essbauer S, Wibbelt G, Isa G, Rudolph M, Gough RE (2004) Avipoxvirus infection in peregrine falcons (*Falco peregrinus*) from a reintroduction programme in Germany. Veterinary Record 154:110-113.

Kruse H, Kirkemo AM, Handeland K (2004) Wildlife as a source of zoonotic infections. Emerging Infectious Diseases 10:2067-2072.

Kumar A, Jindal N, Shukla CL, Asrani RK, Ledoux DR, Rottinghaus GE (2004) Pathological changes in broiler chickens fed ochratoxin A and inoculated with *Escherichia coli*. Avian Pathology 33:413-417.

Lack PC (1986) The Atlas of Wintering Birds in Britain and Ireland. Calton, UK: T. & A.D. Poyser, pp. 1-448.

LaDeau SL, Kilpatrick AM, Marra PP (2007) West Nile virus emergence and large-scale declines of North American bird populations. Nature 447:710-713.

La Ragione RM, McLaren IM, Foster G, Cooley WA, Woodward MJ (2002) Phenotypic and genotypic characterization of avian *Escherichia coli* O86:K61 isolates possessing a Gamma-Like Intimin. Applied and Environmental Microbiology 68:4932-4942.

Lawson B, Jepson P (2004) UK phocine distemper virus epizootic investigation report 2002/2003. Report to the Department for Environment, Food and Rural Affairs, pp. 1-221.

Lawson B (2006a) Garden Bird Health *initiative*. Symposium of the British Wildlife Rehabilitation Council 2006. Cambridge, UK October 2006.

Lawson B, Cunningham A, Chantrey J, Hughes L, Kirkwood J, Pennycott T, Simpson V (2006b) Epidemic finch mortality. Veterinary Record 159:367.

Lederbeg J, Shope RE, Oakes SC (1992) Emerging infections: microbial threat to health in the United States. Washington D.C., U.S.A.: Institute of Medicine, National Academy Press, pp. 1-304.

Le Minor L, Popoff MY (1997) Designation of *Salmonella enteritica* sp. nov., nom. rev., as the type and only species of the genus *Salmonella*. International Journal of Systematic Bacteriology 37:465-468.

LePage D, Francis CM (2002) Do feeder counts reliably indicate bird population changes? 21 years of winter bird counts in Ontario, Canada. The Condor 104:255-270.

Levine ND, Boley LE, Hester HR (1941) Experimental transmission of *Trichomonas gallinae* from the chicken to other birds. American Journal of Hygiene 33:23-32.

Locke LN, Shillinger RB, Jareed T (1973) Salmonellosis in passerine birds in Maryland and West Virginia. Journal of Wildlife Diseases 9:144-145.

Loram A, Tratalos J, Warren PH, Gaston KJ (2007) Urban domestic gardens (X): the extent & structure of the resource in five major cities. Landscape Ecology 22:601–615.

Lüschow D, Hoffman T, Hafez HM (2004) Differentiation of avian poxvirus strains on the basis of nucleotide sequences of 4b gene fragment. Avian Diseases 48:453-462.

Macdonald JW, Cornelius LW (1969) Salmonellosis in wild birds. British Birds 62:28-30.

Macdonald JW, Brown DD (1974) Salmonella infection in wild birds in Britain. Veterinary Record 94:321-322.

Macdonald JW, Bell JC (1980) Salmonellosis in horses and wild birds. Veterinary Record 107:46-47.

Madden UA, Stahr HM (1995) Retention and distribution of aflatoxin in tissues of chicks fed aflatoxin-contaminated poultry rations amended with soil. Veterinary and Human Toxicology 37:24-29.

Maia PP, Pereira Bastos de Siqueira ME (2002) Occurrence of aflatoxins B_1, B_2, G_1 and G_2 in some Brazilian pet foods. Food Additives and Contaminants 19:1180-1183.

Main IG (1996) Seasonal movements of British greenfinches *Carduelis chloris*. Bird Study 43:240-252.

Main IG (1999) Overseas movements to and from Britain by greenfinches *Carduelis chloris*. Ringing and Migration 20:167-180.

Martyn KP (1988) Provisional atlas of the ticks (Ixodoidea) of the British Isles. Biological Records Centre, Monks Wood Experimental Station, Abbots Ripton, Huntingdonshire, UK, pp 1-62.

Mason CF (2000) Thrushes now largely restricted to the built environment in eastern England. Diversity and Distributions 6:189–194.

Mattos A, Solé-Cava AM, DeCarli G, Benchimol M (1997) Fine structure and isozymic characterization of trichomonadidid protozoa. Parasitology Research 83:290-295.

May RM (1988) Control of feline delinquency. Nature 332:392-393.

McClure HE (1989) Epizootic lesions of house finches in Ventura County, California. Journal of Field Ornithology 60:421-430.

McDiarmid A (1969) Diseases in free living wild animals. Symposia of the Zoological Society of London 24. London, UK: Academic Press, pp. 1-332.

McFerran JB, McNulty MS (1993) Poxviridae. In: McFerran JB, McNulty MS, Horzinek MC (Eds.) Virus Infections of Birds. Oxford, UK: Elsevier Scientific Publishing, pp. 1-15.

McKenzie AJ, Petty SJ, Toms MP, Furness R (2007) Importance of Sitka spruce *Picea sitchensis* seed and garden bird-feeders for siskins *Carduelis spinus* and coal tits *Periparus ater*. Bird Study 54:236-247.

Medina FM, Ramirez GA, Hernandez A (2004) Avian pox in white-tailed Laurel-pigeons from the Canary Islands. Journal of Wildlife Diseases 40:351-355.

Met Office (2009) Meteorological Office UK Climate and Weather Statistics. Available: http://www.metoffice.gov.uk/climate/uk/ [Accessed June 2009].

Met Office (2010) Met Office: UK actual and anomaly maps. Available: http://www.metoffice.gov.uk/climate/uk/anomalygraphs/index.html. [Accessed March 2010].

Miller JR (2005) Biodiversity conservation and the extinction of experience. Trends in Ecology and Evolution 20:430-434.

Misof K (2005) Eurasian Blackbirds (*Turdus merula*) and their gastrointestinal parasites: A role for parasites in life-history decisions? PhD thesis. University of Bonn, Germany.

Møller AP, Erritzøe J (2000) Predation against birds with low immunocompetence. Oecologia 122:500-504.

Monks D, Fisher M, Forbes NA (2006) *Ixodes frontalis* and avian tick-related syndrome in the United Kingdom. Journal of Small Animal Practice 47:451-455.

Morgan JAT, Morris GM, Wlodek BM, Bymes R, Jenner M, Constantinoiu CC, Anderson GR, Lew-Tabor AE, Molloy JB, Gasser RB, Jorgensen Wk (2009) Real-time polymerase chain reaction (PCR) assays for the specific detection and quantification of seven Eimeria species that cause coccidiosis in chickens. Molecular and Cellular Probes 23:83-89.

Morishitia TY, Aye PO, Ley EC, Harr BS (1999) Survey of pathogens and blood parasites in free-living passerines. Avian Diseases 43:549-552.

Mörner T, Obendorf DL, Artois M, Woodford MH (2002) Surveillance and monitoring of wildlife diseases. Revue Scientifique et Technique Office des Épizooties 21:67-76.

Mörner AP, Mörner T (1993) Salmonellosis in Swedish wildlife. Proceedings of an International Course on *Salmonella* Control in Animal Production and Products. Mälmo, Sweden, August 1993, pp. 151-154.

Morse SS (1993) Examining the origins of emerging viruses. In: Morse SS (Ed.) Emerging Viruses. New York, U.S.A.: Oxford University Press, pp. 10-28.

Moss S, Cottridge D (1998) Attracting birds to your garden. London, UK: New Holland, pp. 1-160.

Moss S (2000) Bird-Friendly Garden: a practical month-by-month guide to attracting birds to your garden. London, UK: Harper Collins Publishers, pp. 1-144.

Muller RD, Carlson CW, Semeniuk G, Harshfield GS (1970) The response of chicks, ducklings, goslings, pheasants, and poults to graded levels of aflatoxins. Poultry Science 49:1346-1350.

Nadler SA, Honigberg BM (1988) Genetic differentiation and biochemical polymorphism among trichomonads. Journal of Parasitology 74:797-804.

Narcisi EM, Sevolan M, Honigberg BN (1991) Pathologic changes in pigeons infected with a virulent *Trichomonas gallinae* strain (Eiberg). Avian Diseases 35:55-61.

Natural England (2010) Natural England –for people, for places for nature. Available: http://www.naturalengland.org.uk/ [Accessed February 2008].

Neff JA (1955) Outbreak of aspergillosis in mallards. Journal of Wildlife Management 19:415-416.

Neimanis AA, Handeland K, Isomursu M, Ågren E, Mattsson R, Hamnes IS, Bergsjø B, Varpu Hirvelä-Kosk V (2010) First report of epizootic trichomoniasis in wild finches (Family Fringillidae) in southern Fennoscandia. Avian Diseases 51:136-141.

Nemeth NM, Young GR, Burkhalter KL, Brault AC, Reisen WK, Komar N (2009) West Nile virus detection in nonvascular feathers from avian carcasses. Journal of Veterinary Medical Science 21:616-622.

Newson SE, Evans KL, Noble DG, Greenwood JJD, Gaston KJ (2008) Use of distance sampling to improve estimates of national population sizes for common and widespread breeding birds in the UK. Journal of Applied Ecology 45:1330-1338.

Newton I (1972) Finches. London, UK: The New Naturalist, Collins, pp. 1-288.

Nielsen BB, Clausen B (1975) The incidence of *Salmonella* bacteria in Danish wildlife and in imported animals. Nordisk Veterinærmedicin 27:633-640.

Norman D (2002) Chaffinch *Fringilla coelebs*. In: Wernham C, Toms M, Marchant J, Clark J, Siriwardena G, Baille S (Eds.) The Migration Atlas: Movements of the Birds of Britain and Ireland. Thetford, UK: British Trust for Ornithology, pp. 637-640.

NWHC (2002) Quarterly Wildlife Mortality Report October 2002 – December 2002. United States Geological Survey. National Wildlife Heath Center, U.S.A. Available: http://www.nwhc.usgs.gov/publications/quarterly_reports/2002_qtr_4.jsp [Accessed June 2009].

Oaks JL, Beckman KB, Besser TE, Haldorson GS, Bradway DS, Rurangirwa FR, Burek KA (2005) Mortality in redpoll finches in Alaska (*Carduelis flammea*) due to *Escherichia albertii*. Federal Aid Annual Research Report, Alaska Department of Fish and Game Division of Wildlife Conservation, p 9.

Oberheu DG, Dabbert CB (2001a) Aflatoxin contamination in supplemental and wild foods of northern bobwhite. Ecotoxicology 10:125-129.

Oberheu DG, Dabbert CB (2001b) Aflatoxin production in supplemental feeders provided for northern bobwhite in Texas and Oklahoma. Journal of Wildlife Diseases 37:475-480.

Oberheu DG, Dabbert CB (2001c) Exposure of game birds to ochratoxin A through supplemental feeds. Journal of Zoo and Wildlife Medicine 32:136-138.

(ONR) Office for National Statistics (2005) United Kingdom National Census 2001. Available: http://www.statistics.gov.uk/census2001/census2001.asp. [Accessed September 2008].

Oguz H, Kurtoflu V, Coskun B (2000) Preventive efficacy of clinoptilolite in broilers during chronic aflatoxin (50 and 100 ppb) exposure. Research in Veterinary Science 69:197-201.

Oliveira CAF, Rosmaninho JF, Butkeraitis P, Correa B, Reis TA, Guerra JL, Albuquerque R, Moro MEG (2002) Effect of low levels of dietary aflatoxin B1 on laying Japanese quail. Poultry Science 81:976-980.

Orell M (1989) Population fluctuations and survival of great tits *Parus major* dependent on food supplied by man in winter. Ibis 131:112-127.

Ortatatli M, Ciftci MK, Tuzcu M, Kaya A (2002) The effects of aflatoxin on the reproductive system of roosters. Research in Veterinary Science 72:29-36.

Ortatatli M, Oğuz H, Hatipoğlu F, Karaman M (2005) Evaluation of pathological changes in broilers during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure. Research in Veterinary Science 78:61-68.

Osborn D, Nuttall P, Hall A, Harwood J (1990) Wildlife Disease Investigation Review. Swindon, UK: Department of the Environment and Natural Environment Research Council.

Palade EA, Biró N, Dobos-Kovács M, Demeter Z, Mándoki M, Rusvai M (2008) Poxvirus infection in Hungarian great tits (*Parus major*). Acta Veterinaria Hungarica 56:539-546.

Palmgren H, Sellin M, Bergström S, Olsen B (1997) Enteropathogenic bacteria in migrating birds arriving in Sweden. Scandinavian Journal of Infectious Diseases 29:565-568.

Pawiak R, Mazurkiewicz M, Molenda J, Wieliczko A (1991) Nestling mortality of granivorous birds due to microorganisms and toxic substances. Warsaw, Poland: Polish Scientific Publishers, p. 139.

Peighambari SM, Julian RJ, Vaillancourt JP, Gyles CL (1995) *Escherichia coli* cellulitis: experimental infection in broiler chickens. Avian Diseases 39:125-134.

Penfold JB, Amery HC, Peet PJ (1979) Gastroenteritis associated with wild birds in a hospital kitchen. British Medical Journal 2:802.

Pennycott TW (1998) Carriage of trichomonads, *Hexamita* species and *Blastocystis* species by adult pheasants. Veterinary Record 143:142-143.

Pennycott TW, Ross HM, McLaren IM, Park A, Hopkins GF, Foster G (1998a) Causes of death of wild birds of the family Fringillidae in Britain. Veterinary Record 143:155-158.

Pennycott TW, Kirkwood JK (1998b) Feeding wild birds. Veterinary Record 143:371-372.

Pennycott TW (2001) Deaths in finches and sparrows. Available: http://www.bvpa.org.uk/papers/penn01wb.htm [Accessed September 2009].

Pennycott TW, Cinderey RN, Park A, Mather HA, Foster G (2002) *Salmonella enterica* subspecies *enterica* serotype Typhimurium and *Escherichia coli* 086 in wild birds at two garden sites in south-west Scotland. Veterinary Record 151:563-567.

Pennycott TW (2003) Scaly leg, papillomas and pox in wild birds. Veterinary Record 152:444.

Pennycott TW, Lawson B, Cunningham AA, Simpson V, Chantrey J (2005a) Necrotic ingluvitis in wild finches. Veterinary Record 157:360.

Pennycott TW, Cinderey RN, Park A, Mather HA, Foster G, Grant D (2005b) Further monitoring for Salmonella species and *Escherichia coli* 086 at a bird table in south-west Scotland. Veterinary Record 157:477-480.

Pennycott TW, Park A, Mather HA (2006) Isolation of different serovars of *Salmonella enterica* from wild birds in Great Britain between 1995 and 2003. Veterinary Record 158:817-820.

Pennycott TW, Dalgleish MP, Wood AM, Garcia C (2009) *Chlamydophila psittaci* in wild birds in the UK. Veterinary Record 164:157-158.

Perez M, Henke SE, Fedynich AM (2001) Detection of aflatoxin-contaminated grain by three granivorous bird species. Journal of Wildlife Diseases 37:358-361.

Perez-Mesa C, Stabler RM, Berthrong M (1961) Histopathological changes in the domestic pigeon infected with *Trichomonas gallinae* (Jones-Barn strain). Avian Diseases 5:48-60.

Peters M, Kilwinski J, Reckling D, Henning K (2009) Gehäufte Todesfälle von wild lebenden Grünfinken an Futterstellen infolge *Trichomonas-gallinae*-Infektionen – ein aktuelles Problem in Norddeutschland. Kleintierpraxis 54:433–438.

Peterson AT, Vieglais DA, Andreasen JK (2003) Migratory birds modeled as critical transport agents for West Nile Virus in North America. Vector Borne and Zoonotic Diseases 3:27-37.

Petrie A, Sabin C (2000) Medical statistics at a glance. Oxford, UK: Blackwell Science Ltd., pp. 1-138.

Philbey AW, Mather HA, Taylor DJ, Coia JE (2008) Isolation of avian strains of *Salmonella enterica* serovar Typhimurium form cats with enteric disease in the United Kingdom. Veterinary Record 162:120-122.

Philbey AW, Brown FM, Mather HA, Coia JE, Taylor DJ (2009) Salmonellosis in cats in the United Kingdom: 1955 to 2007. Veterinary Record 164:120-122.

Pier AC, Heddleston KL (1970) The effect of aflatoxin on immunity in turkeys. I. Impairment of actively acquired resistance to bacterial challenge. Avian Diseases 14:797-809.

Pier AC (1992) Major biological consequences of aflatoxicosis in animal production. Journal of Animal Science 70:3964-3967.

Pitt JI, Hocking AD (1997) Fungi and food spoilage. London, UK: Blackie Academic & Professional, pp. 1-540.

Plumb J (2008) Deaths in juvenile hedgehogs. Veterinary Record 163:549-550.

Pocock MJO, Searle JB, Betts WB, White PCL (2001) Patterns of infection by *Salmonella* and *Yersinia* spp. in commensal house mice (*Mus musculus domesticus*) populations. Journal of Applied Microbiology 90:755-760.

Pokras MA, Wheeldon EB, Sedgwick CJ (1993) Trichomoniasis in owls: report on a number of clinical cases and a survey of the literature. In: Redig PT, Cooper JE, Remple JD, Hunter DB (Eds.) Raptor Biomedicine. Minneapolis, Minnesota, U.S.A.: University of Minnesota Press, pp. 88-91.

Polowinkin P (1901) Beitrag zur pathologischen Anatomis der Taubenpocke. Archives Tierheikunde 27:86-109.

Poulding RH (1960) Fowlpox in a carrion crow. British Birds 53:174-175.

Prescott JF, Poppe C, Goltz J, Campbell GD (1998) *Salmonella typhimurium* phage type 40 in feeder birds. Veterinary Record 142:732.

Project FeederWatch (2009) Project FeederWatch. Available: http://www.birds.cornell.edu/pfw/ [Accessed January 2010].

Quist CF, Bounous DI, Kilburn JV, Nettles VF, Wyatt RD (2000) The effect of dietary aflatoxin on wild turkey poults. Journal of Wildlife Diseases 36:436-444.

Qureshi MA, Brake J, Hamilton PB, Hagler WM, Nesheim S (1998) Dietary exposure of broiler breeders to aflatoxin results in immune dysfunction in progeny chicks. Poultry Science 77:812-819.

Rabsch W, Andrews HL, Kingsley RA, Prager R, Tschäpe H, Adams LG, Bäumler AJ (2002) *Salmonella enterica* Serotype Typhimurium and its host-adapted variants. Infection and Immunity 70:2249-2255.

Ramsden DJ (2003) Barn owls and major roads: results and recommendations from a 15-year research project.Waterleat, UK: The Barn Owl Trust, pp.1-112.

Randall CJ, Reece RL (1996) Colour Atlas of Avian Histopathology. London, UK: Mosby-Wolfe, pp. 1-232.

Rappole JH, Derrickson SR, Hubálek Z (2000) Migratory birds and spread of West Nile virus in the Western Hemisphere. Emerging Infectious Diseases 6:319-328.

Raven MJ, Noble DG, Baillie SR (2004) The Breeding Bird Survey 2003. BTO Research Report 363. Thetford, UK: British Trust for Ornithology, pp. 1-17.

R Development Core Team (2007) R: a language and environment for statistical computing. Available: http://www.R-project.org [Accessed June 2008].

Real J, Mañosa S, Muñoz E (2000) Trichomoniasis in a Bonelli's eagle population in Spain. Journal of Wildlife Diseases 36:64-70.

Redfern CPF, Clark JA (2001) The ringers' manual. Thetford, UK: British Trust for Ornithology, pp. 1-270.

Reed TE, Daunt F, Hall ME, Phillips RA, Wanless S, Cunningham EJA (2008) Parasite treatment affects maternal investment in sons. Science 321:1681-1682.

Reeve N (1994) Hedgehogs. London, UK: Poyser Natural History, pp. 1-313.

Refsum T, Handeland K, Baggesen DL, Holstad G, Kapperud G (2002a) Salmonellae in avian wildlife in Norway from 1969 to 2000. Applied and Environmental Microbiology 68:5595-5599.

Refsum T, Heir E, Kapperud G, Vardund T, Holstad G (2002b) Molecular epidemiology of *Salmonella enterica* Serovar Typhimurium isolates determined by pulsed-field gel electrophoresis: comparison of isolates from avian wildlife, domestic animals, and the environment in Norway. Applied and Environmental Microbiology 68:5600-5606.

Refsum T, Vikøren T, Handeland K, Kapperud G, Holstad G (2003) Epidemiologic and pathologic aspects of *Salmonella typhimurium* infection in passerine birds in Norway. Journal of Wildlife Diseases 39:64-72.

Rickards L, Fox K, Roberts C, Fletcher L, Goddard E (2004) Living in Britain: Results from the 2002 General Household Survey. General health and use of health services. London, UK: Office for National Statistics, pp. 1-299.

Risely K, Noble DG, Baillie SR (2008) The Breeding Bird Survey 2007, BTO Research Report 508. Thetford, UK: British Trust for Ornithology, pp. 1-20.

Risely K, Noble DG, Baillie SR (2009) The Breeding Bird Survey 2008, BTO Research Report 537. Thetford, UK: British Trust for Ornithology, pp. 1-24.

Ritchie BW (1995) Poxviridae. In: Ritchie BW (Ed.) Avian viruses – Function and Control. Lake Worth, U.S.A.: Wingers Publishing Inc., pp. 285-311.

Rivolta S (1878) Una forma di croup prodotta da un infusorio, ni olli. Gionrale di Anatomia, Fisiologia, e Patologia Animmale 10:149-158.

Robb GM, McDonald RA, Chamberlain DE, Bearhop S (2008) Food for thought: supplementary feeding as a driver of ecological change in avian populations. Frontiers in Ecology and the Environment 6:476-484.

Robinson RA, Daniel MJ (1968) The significance of salmonella isolates from wild birds and rats in New Zealand. New Zealand Veterinary Journal 16:53-55.

Robinson RA, Siriwardena GM, Humphrey QPC (2005) Size and trends of the house sparrow *Passer domesticus* in Great Britain. Ibis 147:552-562.

Robinson RA, Baillie SR, Crick HQP (2007) Weather-dependent survival: implications of climate change for passerine population processes. Ibis 149:357-364.

Robinson RA. Grantham MJ, Clark JA (2009) Declining rates of ring recovery in British birds. Ringing & Migration 24:266-272.

Robinson RM, Ray AC, Reagor JC, Holland LA (1982) Waterfowl mortality caused by aflatoxicosis in Texas. Journal of Wildlife Diseases 18:311-312.

Routh A, Sleeman JM (1995) Greenfinch mortalities. Veterinary Record 136:500.

Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (Eds.) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, New Jersey, U.S.A.: Humana Press, pp. 365-386.

(RSPB) Royal Society for the Protection of Birds (2004) Feeding Garden Birds Survey. RSPB Report 0411, pp. 1-13.

RSPB (2008) Homes for Wildlife. Available: http://www.rspb.org.uk/hfw/about/index.asp [Accessed January 2010].

RSPB (2009) A to Z of a wildlife garden: peanut feeder. Available: http://www.rspb.org.uk/wildlife/wildlifegarden/atoz/p/peanutfeeder.aspx [Accessed February 2010].

Ruff MD, Huff WE, Wilkins GC (1990) Characterisation of the toxicity of the mycotoxins aflatoxin, ochratoxin, and T-2 Toxin in game birds. I. Chukar Partridge. Avian Diseases 34:717-720.

Ruff MD, Huff WE, Wilkins GC (1992) Characterisation of the toxicity of the mycotoxins aflatoxin, ochratoxin, and T-2 Toxin in game birds. III. Bobwhite and Japanese Quail. Avian Diseases 36:34-39.

Sainsbury AW, Kirkwood JK, Bennett PM, Cunningham AA (2001) Status of wildlife health monitoring in the United Kingdom. Veterinary Record 148:558-563.

Sainsbury AW, Deaville R, Lawson B, Cooley WA, Farelly SS, Stack MJ, Duff P, McInnes CJ, Gurnell J, Russell PH, Rushton SP, Pfeiffer DU, Nettleton P, Lurz PW (2008) Poxviral disease in red squirrels Scirurus vulgaris in the UK: spatial and temporal trends of an emerging threat. Ecohealth 5:305-16.

Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406-425.

Salunkhe DK, Adsule RN, Padule DN (1987) Aflatoxins in foods and feeds. New Delhi, India: Metropolitan Book Co., pp. 1-510.

Samuel MD, Botzler RG, Wobeser GA (2007) Avian cholera. In: Thomas NJ, Hunter DB, Atkinson CT (Eds.) Infectious Diseases of Wild Birds. Oxford, UK: Wiley-Blackwell Publishing, pp. 239-269.

Sandmeier P, Couttell P (2006) Management of canaries, finches and mynahs. In: Harrison GJ, Lightfoot TL (Eds.) Clinical Avian Medicine Volume II. Palm Beach, Florida, U.S.A.: Spix Publishing, pp. 880-914.

Sansano J, Garijo-Toledo MM, Gomez-Munoz MT (2008) Genetic characterization of *Trichomonas gallinae* indicated correlation with its pathogenicity. Poster presented at the European Wildlife Disease Association Conference, Rovinj, Croatia, October 2008.

Santin E (2005) Mould growth and mycotoxin production. In: Diaz D (Ed.) The Mycotoxin Blue Book. Nottingham, UK: Nottingham University Press, pp. 225-234.

Schaal E, Ernst H (1967) Enzootic occurrence of salmonellosis in local wild birds. Berliner und Münchener Tierärtzliche Wochenschrift 80:13-16.

Schrenzel MD, Mallouf GA, Gaffney PM, Tokarz D, Keener LL, McClure D, Griffey S, McAloose D, Rideout BA (2005). Molecular characterization of isosporoid coccidian (*Isospora* and *Atoxoplasma* spp.) in passerine birds. Journal of Parasitology 91:635-647.

Schweitzer SH, Quist CF, Grimes GL, Forster DL (2001) Aflatoxin levels in corn available as wild turkey feed in Georgia. Journal of Wildlife Diseases 37:657-659.

Scott FW (1988) Salmonella implicated as a cause of song bird fever. Feline Health Topics for Veterinarians, Cornell Feline Health Centre 3:506.

Scudamore KA, Hetmanski MT, Nawaz S, Naylor J, Rainbird S (1997) Determination of mycotoxins in pet foods sold for domestic pets and wild birds using linked-column immunoassay clean-up and HPLC. Food Additives and Contaminants 14:175-186.

Sharma RP (1993) Immunotoxicity of Mycotoxins. Journal of Dairy Science 76:892-897.

Sharman M, Gilbert J (1991) Automated aflatoxin analysis of foods and animal feeds using immunoaffinity column clean-up and high-performance liquid chromatographic determination. Journal of Chromatography 543:220-225.

Sharman M, MacDonald S, Gilbert J (1992) Automated liquid chromatographic determination of ochratoxin A in cereals and animal products using immunoaffinity column clean-up. Journal of Chromatography 603:285-289.

Sheldon BC, Verhulst S (1996) Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends in Ecology and Evolution 11:317-321.

Shi YH, Xu ZR, Feng JL, Wang CZ (2006) Efficacy of modified montmorillonite nanocomposite to reduce the toxicity of aflatoxin in broiler chicks. Animal Feed Science and Technology 129:138-148.

Shotwell OL (1991) Natural occurrence of mycotoxins in corn. In: Smith JE, Henderson RS (Eds.) Mycotoxins and Animal Foods. Boca Raton, Florida, U.S.A.: CRC Press Inc., pp. 325-340.

Simpson CE, Forrester DL, Nesbitt SA (1975) Avian pox in Florida Sandhill cranes. Journal of Wildlife Diseases 11:112-115.

Simpson VR (1988) Wildlife health – who cares? British Veterinary Zoological Society Newsletter 27:12-15.

Simpson VR, Bevan R (1989) *Chlamydia psittaci* infection in robins. 125:537.

Simpson VR, Bain MS, Brown BF, Lacey RF (2000) A long-term study of vitamin A and polychlorinated hydrocarbon levels in otters (*Lutra lutra*) in south west England. Environmental Pollution 110:267-275.

Simpson VR (2002) Wild animals as reservoirs of infectious diseases in the UK. The Veterinary Journal 163:128-146.

Simpson VR (2006) Patterns and significance of bite wounds in Eurasian otters (Lutra lutra) in southern ad south-west England. Veterinary Record 158:113-119.

Skov MN, Madsen JJ, Rahbek C, Lodal J, Jespersen JB, Jørgensen JC, Dietz HH, Chriél M, Baggesen DL (2008) Transmission of Salmonella between wildlife and meat-production animals in Denmark. Journal of Applied Microbiology 105:1558-1568.

Smith JE, Ross K (1991) The toxigenic aspergilli. In: Smith JE, Henderson RS (Eds.) Mycotoxins and Animal Foods. Boca Raton, Florida, U.S.A.: CRC Press Inc., pp. 101-118.

Smith RT, Eitzman DV (1964) The development of the immune response. Characterisation of the response of the human infant and adult to immunization with salmonella vaccines. Pediatrics 33:163-183.

Smits JE, Tella JL, Carrete M, Serrao D, Lopez G (2005) An epizootic of avian pox in endemic short-toed larks (*Calandrella rufescens*) and Berthelot's Pipits (*Anthus berthelotti*) in the Canary Islands Spain. Veterinary Pathology 42:59-65.

Snow DW, Perrins CM (1998) The birds of the western Palearctic, concise edition; Vol. 2. Oxford, UK: Oxford University Press, p. 1694.

Sojka WJ (1965) *E. coli* in domestic animals and poultry. Commonwealth Agricultural Bureau, Wallingford, Oxfordshire, UK.

Soll DR (2000) The ins and outs of DNA fingerprinting the infectious fungi. Clinical Microbiology Reviews 13:332-370.

Soulsby EJL (1968) Helminths, Arthropods and Protozoa of Domesticated Animals. London, UK: Balliere, Tindall and Cassell, pp. 583-586.

Speek G, Clark J, Rhode Z, Wassenaar RD, Van Noordwijk AJ (2001) The Euring Exchange code 2000. Heteren, Netherlands: Euring, pp. 1-143.

Stabler RM (1938) *Trichomonas gallinae* (Rivolta, 1878) the correct name for the flagellate in the mouth, crop and liver of the pigeon. Journal of Parasitology 24:553-554.

Stabler RM (1954) *Trichomonas gallinae*: a review. Experimental Parasitology 3:368-402.

Stewart RG (1985) Natural exposure of bobwhite quail to aflatoxin. PhD thesis. University of Georgia, Athens, Georgia, U.S.A.

Stroud RK, Duncan RM (1982) Occlusion of the syrinx as a manifestation of aspergillosis in Canada geese. Journal of the American Veterinary Medical Association 181:1389-1390.

Summers-Smith JD (1963) The House Sparrow. London, UK: The New Naturalist, Collins, pp. 1-269.

Sweeney MJ, Dobson ADW (1998) Mycotoxin production by *Aspergillus*, *Fusarium* and *Penicillium* species. International Journal of Food Microbiology 43:141-158.

Tamási G (1981) Factors influencing the survival of pathogenic bacteria in soils. Journal Acta Veterinaria Academiae Scientiarum Hungaricae 29:119-126.

Tamura K, Nei M, Kumar S (2004) Prospects for inferring very large phylogenies by using the neighbor-joining method. Proceedings of the National Academy of Sciences (USA) 101:11030-11035.

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24:1596-1599.

Tauni MA, Österlund A (2000) Outbreak of *Salmonella typhimurium* in cats and humans associated with infection in wild birds. Journal of Small Animal Practice 41:339-341.

Taylor J (1968) Salmonella in wild animals. Symposium of the Zoological Society of London 24:51-73.

Thompson C, Henke SE (2000) Effect of climate and type of storage container on aflatoxin production in corn and its associated risks to wildlife species. Journal of Wildlife Diseases 36:172-179.

Thornley CN, Simmons GC, Callaghan ML, Nicol CM, Baker MG, Gilmore KS, Garrett NKG (2003) First incursion of *Salmonella enterica* Serotype Typhimurium DT160 into New Zealand. Emerging Infectious Diseases 9:493-495.

Threlfall EJ, Ward LR, Rowe B (1978) Epidemic spread of a Chloramphenicol-resistant strain of *Salmonella Typhimurium* phage type 204 in bovine animals in Britain. Veterinary Record 103:438-440.

Thrusfield M (1995) Veterinary Epidemiology. Oxford, UK: Blackwell Science Ltd., pp. 26-27.

Tizard IR, Fish NA, Harmeson J (1979) Free flying sparrows as carriers of Salmonellosis. Canadian Veterinary Journal 20:143-144.

Tizard IR (2004) Salmonellosis in wild birds. Seminars in Avian and Pet Medicine. 13:50-66.

Tobin ME, Dolbeer RA (1990) Disappearance and recoverability of songbird carcasses in fruit orchards. Journal of Field Ornithology 61:237-242.

Tompkins D, Sainsbury A, Nettleton P, Buxton D, Gurnell J (2002) Parapoxvirus causes a deleterious disease in red squirrels associated with UK population declines. Proceedings of the Royal Society, London Series B 269:529-533.

Toms MP (2003) The BTO/CJ Garden BirdWatch Book. Thetford, UK: British Trust for Ornithology, pp. 1-128.

Toms MP (2007) Are gardens good for birds or birdwatchers? British Wildlife 19:77-83.

Tripathy DN, Schnitzlein WM, Morris PJ, Janssen DL, Zuba JK, Massey G, Atkinson CT (2000) Characterization of poxviruses from forest birds in Hawaii. Journal of Wildlife Diseases 36:225-230.

Trucksess MW, Stoloff L (1979) Extraction, clean-up, and quantitative determination of aflatoxins B1 and M1 in beef liver. Journal of the Association of Official Analytical Chemists 62:1080-1082.

Trucksess MW, Stoloff L, Young K (1983) Aflatoxicol and Aflatoxins B_1 and M_1 in eggs and tissues of laying hens consuming aflatoxin-contaminated feed. Poultry Science 62:2176-2181.

Trumbell DJ, Bonney R, Bascom D, Cabral A (2000) Thinking scientifically during participation in a citizen-science project. Science Education 84:265-275.

Tsiodras S, Kelesidis T, Kelesidis I, Bauchinger U, Flagas ME (2008) Human infections associated with wild birds. Journal of Infection 56:83-98.

Une Y, Sanbe A, Suzuki S, Niwa T, Kawakami K, Kurosawa R, Izumiya H, Watanabe H, Kato Y (2008) Salmonella enterica serotype Typhimurium ifection causing morality in Eurasian tree sparrows (*Passer montanus*) in Hokkaido. Japanese Journal of Infectious Diseases 61:166-167.

UFAW (2005) Feeding garden birds: best practice guidelines., Wheathampstead, UK: Universities Federation for Animal Welfare, pp.1-31.

Väisänen RA, Koskimies P (1991) Monitoring bird populations. A manual of methods applied in Finland. Helsinki, Finland: Finnish Museum of Natural History, University of Helskini.

Vanácová S, Tachezy J, Kulda J, Flegr J (1997) Characterization of trichomonad species and strains by PCR fingerprinting. The Journal of Eukaryote Microbiology 44:545-552.

van der Merwe KJ, Steyn PS, Fourie L, Scott De B, Theron JJ (1965) Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. Nature 205:1112 – 1113.

van Riper C III, van Riper SG, Goff ML, Laird M (1986) The epizootiology and ecological significance of malaria in Hawaiian (USA) land birds. Ecological Monographs 56:327-344.

van Riper C III, van Riper SG, Hansen WR (2002) Epizootiology and effect of avian pox on Hawaiian forest birds. The Auk 199:929-942.

van Riper C III, Forrester DJ (2007) Avian Pox. In: Thomas NJ, Hunter DB, Atkinson CT (Eds.) Infectious Diseases of Wild Birds. Oxford, UK: Wiley-Blackwell Publishing, pp. 131-176.

Vargas H (1987) Frequency and effect of pox-like lesions in Galapagos mockingbirds. Journal of Field Ornithology 58:101-102.

Verma J, Johri TS, Swain BK, Ameena S (2004) Effect of graded levels of aflatoxin, ochratoxin and their combinations on the performance and immune response of broilers. British Poultry Science 45:512-518.

(VLA) Veterinary Laboratories Agency (2005) Salmonella in Livestock Production in GB 2004. Department for the Environment, Food and Rural Affairs, pp. 1-161.

VLA (2006) Wildlife Diseases in the UK Reported in the Year 2007. Report to the Department for the Environment, Food and Rural Affairs and the Office International des Epizooties (OIE), p. 7.

VLA (2007) Veterinary Laboratories Agency Wildlife Surveillance Quarterly Report 9(1), April – June 2007., p. 3.

VLA (2008) Veterinary Laboratories Agency Wildlife Disease Reports. Available: http://www.defra.gov.uk/vla/reports/rep_wildlife.htm [Accessed January 2010].

Vickery JA, Bradbury RB, Henderson IG, Eaton MA, Grice PV (2004) The role of agri-environment schemes and farm management practices in reversing the decline of farmland birds in England. Biological Conservation 119:19-39.

Villanúa D, Höfle U, Pérez-Rodriguez L, Gortázar C (2006) *Trichomonas gallinae* in wintering Common Wood Pigeons *Columba palumbus* in Spain. Ibis 148:641-618.

Viscogliosi E, Durieux I, Delgado-Viscoglisosi P, Bayle D, Dive D (1996) Phylogenetic implication of iron-containing superoxide dismutase genes from trichomonad species. Molecular and Biochemical Parasitology 80:209-214.

Viscogliosi E, Müller M (1998) Phylogenetic relationships of the glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase, from Parabasalid flagellates. Journal of Molecular Ecology 47:190-199.

Voncken FGJ, Boxma B, van Hoek AHAM, Akhmanova AS, Vogels GD, Huynen M, Veenhuis M, Hackstein JHP (2002) A hydrogenosomal [Fe]-hydrogenase from the anaerobic chytrid Neocalllimastix sp. L2. Gene 284:103-112.

Walker LA, Simpson VR, Rockett L, Wienburg CL, Shore RF (2007) Heavy metal contamination in bats in Britain. Environmental Pollution148: 483-490.

Walker RL, Hayes DC, Sawyer SJ, Nordhausen RW, Van Hoosear KA, BonDurant RH (2003) Comparison of the 5.8S rRNA gene and internal transcribed spacer regions of trichomonadid protozoa recovered from the bovine preputial cavity. Journal of Veterinary Diagnostic Investigation 15:14-20.

Ward MR, Stallknecht DE, Willis J, Conroy MJ, Davidson WR (2006) Wild bird mortality and West Nile virus surveillance: biases associated with detection, reporting, and carcass persistence. Journal of Wildlife Diseases 42:92-106.

Warner RE (1968) The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. Condor 70:101-120.

Weli SC, Okeke MI, Tryland M, Nilssen O, Traavik T (2004a) Characterization of avipoxviruses from wild birds in Norway. Canadian Journal of Veterinary Research 68:140-145.

Weli SC, Traavik T, Tryland M, Coucheron DH, Nilssen O (2004b) Analysis and comparison of the 4b core protein gene of avipoxviruses from wild birds: evidence for interspecies spatial phylogenetic variation. Archives of Virology 149:2035-2046.

Wells JV, Rosenberg KV, Dunn EH, Tessagli-Hymers DL, Dhondt AA (1998) Feeder counts as indicators of spatial and temporal variation in winter abundance of resident birds. Journal of Field Ornithology 69: 577-586.

Wernham C, Toms M, Marchant J, Clark J, Siriwardena G, Baille S (2002) The Migration Atlas: Movements of the Birds of Britain and Ireland. Thetford, UK: British Trust for Ornithology, pp. 1-900.

West N (2009) New bird pox threat to great tits and aviaries. Cage and Aviary Birds. October 29, p. 4.

Westerkov K (1953) Bird pox in a New Zealand Pipit (*Anthys novaeseelandiae*). Notornis 5:168-170.

Wheeldon EB, Sedgwick CJ, Schulz TA (1985) Epornitic of avian pox in a raptor rehabilitation center. Journal of the American Veterinary Medical Association 187:1202-1204.

Wigley P, Berchieri A, Page KL, Smith AL, Barrow PA (2001) *Salmonella enterica* serovar Pullorum persists in splenic macrophages and in the reproductive tract during persistent, disease-free carriage in chickens. Infection and Immunity 69:7873-7879.

Wilson JE, Macdonald JW (1967) Salmonella infection in wild birds. British Veterinary Journal 123:212-219.

Wilson JS, Hazel SM, Williams NJ, Phiri A, French NP, Hart CA (2003) Nontyphoidal Salmonellae in United Kingdom Badgers: prevalence and spatial distribution. Applied and Environmental Microbiology 69:4312-4315.

Wobeser GA, Finlayson MC (1969) *Salmonella typhimurium* infection in house sparrows. Archives of Environmental Health 19:882-884.

Wobeser G, Wobeser AG (1992) Carcass disappearance and estimation of mortality in a simulated die-off of small birds. Journal of Wildlife Diseases 28:548-554.

Wood SN (2006) Generalized additive models: an introduction with R. London, UK: Chapman & Hall/ CRC, pp. 1-392.

Woods M, Mcdonald RA, Harris S (2003) Predation of wildlife by domestic cats *Felis catus* in Great Britain. Mammal Review 33:174-188.

Wyatt RD (2005) Mycotoxin interactions. In: Diaz D (Ed.) The Mycotoxin Blue Book. Nottingham, UK: Nottingham University Press, pp. 269-278.

Yakimoff WL, Gouseff WF (1938) Les coccidies du pinson (*Fringilla coelebs* L.) Annales de la Société Belge de Médecine Tropicale 18:523-525.

Yamamato Y, Nakamura K, Yamada M, Ito T (2009) Zoonotic risk for influenza A (H5N1) infection in wild swan feathers. Journal of Veterinary Medical Science 71:1549-1551.

Yoshizawa T (1991) Natural occurrence of mycotoxins in small grain cereals (wheat, barley, rye, oats, sorghum, millet, rice). In: Smith JE, Henderson RS (Eds.) Mycotoxins and Animal Foods. Boca Raton, Florida, U.S.A.: CRC Press, Inc., pp. 301-324.

Ytrehus B (2009) Wildlife Disease Association - Nordic Section Quarterly Report 2009. Available: http://www.medvetnet.org/wildlist/Newsletter_WDA_Oct_09.pdf. [Accessed December 2009].

LIST OF APPENDICES

PUBLISHED PAPERS SUBMITTED IN SUPPORT OF THIS THESIS

Pennycott TW, **Lawson** B, Cunningham AA, Simpson V, Chantrey J (2005) Necrotic ingluvitis in wild finches. Veterinary Record 157:360.

Lawson B, MacDonald SJ, Macgregor SK, Howard TE, Cunningham AA (2006) Exposure of garden birds to aflatoxins in Britain. Science of the Total Environment 361(1-3):124-131.

Lawson B, Cunningham A, Chantrey J, Hughes L, Kirkwood J, Pennycott T, Simpson V (2006) Epidemic finch mortality. Veterinary Record 159:367.

Hughes LA, Shopland S, Wigley P, Bradon H, Leatherbarrow AH, Williams NJ, Bennett M, de Pinna E, **Lawson** B, Cunningham AA, Chantrey J (2008) Characterisation of *Salmonella* enterica serotype Typhimurium isolates from wild birds in northern England from 2005 - 2006. BMC Veterinary Research 29(4):4.

Robinson RA, **Lawson** B, Toms MP, Peck KM, Kirkwood JK, et al. (2010) Emerging Infectious Disease Leads to Rapid Population Declines of Common British Birds. PLoS ONE 5(8): e12215. doi:10.1371/journal.pone.0012215

Lawson B, Malnick H, Pennycott TW, Macgregor SK, John SK, Duncan G, Hughes LA, Chantrey J, Cunningham AA (*in press*) Acute necrotising pneumonitis associated with *Suttonella ornithocola* infection in tits (Paridae). The Veterinary Journal.

AM number: .....

GBH*i* AVIAN MORTALITY (AM) QUESTIONNAIRE

AM Number (IOZ/ LIV/ SAC/ WVIC/ RSPB-AM-0001-YR): ....
Date reported: .....
Reporter agrees with data protection statement? Yes ☐ No ☐
Name of person making the report: .....
Address: .....
.....
.....**Postcode:** .....
Telephone number (Single contact number, others taken if required):
Home: ..... **Work:** .....
Mobile: .....
Fax: .....
Email: .....

Member of BTO/CJ Garden BirdWatch? Yes ☐ No ☐
If yes, GBW number:.....

Species involved 1: .....
Date problem was first noticed (sick or dead birds)? .....
Number sick: .....
Number dead: .....
Clinical signs observed?

Fluffed up	☐	Lethargic	☐	Unable to fly	☐
Still trying to feed	☐	Gasping	☐	Difficulty in swallowing	☐

Other .....
.....

Species involved 2: .....
Date problem was first noticed (sick or dead birds)? .....
Number sick: .....
Number dead: .....
Clinical signs observed?

Fluffed up	☐	Lethargic	☐	Unable to fly	☐
Still trying to feed	☐	Gasping	☐	Difficulty in swallowing	☐

Other .....
.....

Species involved 3: .....
Date problem was first noticed (sick or dead birds)? .....
Number sick: .....
Number dead: .....
Clinical signs observed?

Fluffed up	☐	Lethargic	☐	Unable to fly	☐
Still trying to feed	☐	Gasping	☐	Difficulty in swallowing	☐

Other .....
.....

AM number: .....

Brief notes on the circumstances (e.g. location where bird was found, near feeding table, possible predation, collision with window, poisoning, road accident, etc):

.....
.....
.....

Have you had any outbreaks of disease in the past? Yes ☐ No ☐
If so, when? .....
If so, which species were affected? .....
.....
.....

What is the size of your garden?

Small ☐ Medium ☐ Large ☐

Small refers to gardens less than 100 square metres (120 square yards) or about half a tennis court in size.
Medium refers to gardens between 100 and 450 square metres (120-450 square yards) or between half a tennis court and two courts in size.
Large refers to gardens more than 450 square metres (540 square yards) or larger than two tennis courts in size.
1 acre ~ 4000 square metres 1 hectare ~ 2.5 acres =10,000 square metres

.....
.....

Type of land use around the garden?

Rural ☐ Suburban ☐ Urban ☐

Recent weather?

Average for season ☐ High winds ☐ Extreme cold ☐
Very rainy ☐ Snow cover ☐ Heavy frost ☐
Heat wave ☐ Drought ☐
Other .....

Do you feed birds in the garden?

Never ☐ Rarely ☐ Sometimes (~monthly) ☐
Weekly ☐ Daily ☐

At what time of year do you tend to feed birds?

Winter only ☐ Year round ☐ Variable ☐

Where do you purchase food (seed and peanuts) for the birds?

Mail order ☐ Pet shop ☐ Supermarket ☐
Feed merchant ☐ Garden centre ☐ Other .....

Which type of seed &/or peanuts do you purchase?

Non-branded only ☐
Branded only ☐
Mix of above ☐
Brand Name(s): .....

AM number: .....

How often do you provide the following food types?

Food type	Most of the time (regularly)	Sometimes (intermittently)	Never
Wild bird seed mix			
Sunflower seeds			
Sunflower seed hearts			
Peanuts			
Niger seed			
Fat balls			
Household scraps			
Live foods			
Other			

How much food (mixed bird seed/ sunflower seed/ peanuts) do you currently provide in your garden each day?

----------g/ DAY

Estimate the amount wherever possible; otherwise select one of the following three groups.

Less than 500 g/ DAY ☐ 500-2000 g/ DAY ☐ > 2000 g/ DAY ☐

Confidence of reporter in accuracy of the estimate?

High confidence ☐ Moderate confidence ☐ Low confidence ☐

Cup ~ 250 ml Pint ~ 570 ml ~ 500 g

Pound = 454 g Ounce = 28 g Hundredweight ~ 50kg

What types of feeder are used?

Type of feeder	Number	Details
Hanging		
Table		
Ground		
Other		
Water bath		

.....

.....

.....

Are the feeders in your garden in clustered in one area or spread throughout the garden?

Clustered ☐ Spread throughout garden ☐ Other ☐

Do any of your neighbours feed birds in their gardens?

None ☐ Some (1-3) ☐ Many (4+) ☐ Don't know ☐

AM number: .....

How often do you clean your bird table?
Daily ☐ Weekly ☐ Monthly ☐ Less frequently ☐ Never ☐ N/A ☐

How often do you clean your hanging feeders?
Daily ☐ Weekly ☐ Monthly ☐ Less frequently ☐ Never ☐ N/A ☐

Do you use cleaning agents when cleaning feeders/ bird tables? Yes ☐ No ☐
Detergent used? Yes ☐ No ☐
Disinfectant used? Yes ☐ No ☐
Product names and other details?.....
.....
.....
.....
.....

Other bird species currently seen in the garden (approximate numbers observed in garden)?

Species	**Estimate number**
Blackbird	
Blue tit	
Chaffinch	
Collared dove	
Feral pigeon	
Goldfinch	
Great tit	
Greenfinch	
House sparrow	
Magpie	
"Seagull"	
Siskin	
Sparrowhawk	
Starling	
Wood pigeon	

Other relevant details e.g. member of RSPB, BTO, BTO licensed ringer, cage bird aviary (indoor or outdoor) or domestic poultry nearby, etc.
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AM number: .....

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Where did the reporter hear of the GBHi?

.....
.....

What other organisations/ companies have been informed?

.....
.....

Carcase(s) to be sent? **Yes** ☐ **No** ☐

Species	Carcase Number	PM Number	Date found

Have any other birds been submitted for PME? **Yes** ☐ **No** ☐
Post mortem no(s): .....

Leaflets sent? Yes ☐.....**Date:**.....

Have the post mortem results been reported to the finder?
Yes ☐.....**Date:** .....
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Feedback on the effect of control measures on garden bird morbidity and mortality (duration, total number of birds affected, success of control measures used?)
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GUIDELINES FOR HANDLING AND POSTING DEAD BIRDS FOR POST-MORTEM

Naturally, there are strict rules that govern how specimens must be packaged for postage. It is important that these simple instructions are followed to prevent specimens being destroyed or to avoid any public health risk hazards that may arise following handling of improperly packaged specimens. The Post Office only permits members of the public to submit specimens through the post under the direction of a veterinary surgeon or registered laboratory. Please call your regional laboratory to confirm arrangements before submission of each specimen. Ideally, carcasses should be stored refrigerated but only if you have the facilities to do this (e.g. at a wildlife centre or veterinary surgery). Do not use your domestic refrigerator – carcasses may carry dangerous infectious agents, some of which may affect humans and pets. Carcasses should not be frozen. If there is likely to be a delay before the carcass can be submitted, specific arrangements should be discussed with the regional laboratory.

Any direct contact with the bird should be avoided, i.e. place your hand within a plastic bag, pick up the carcass with the covered hand and then invert the bag over the carcass and seal the bag securely. Please use a separate bag for each carcass. Please wash your hands after packaging the bird.

This bag must then be wrapped in an absorbent material such as cotton wool, absorbent paper or cellulose wadding. Sufficient absorbent material must be used to absorb all possible leakage in the event of damage. This must then be placed within another plastic bag (e.g. freezer zip-loc bag) and sealed to produce a leak-proof package.

The package should then be placed within a rigid, crush proof container (e.g. sturdy plastic tub & clip-down lid/ Tupperware or strong cardboard box with full depth lid) with the lid firmly fixed using self-adhesive tape. This does not have to be done separately for each bird, as long as each specimen is properly bagged.

This container should then be packaged, along with the accompanying completed GBW SUBMISSION SLIP, within a 'jiffy-type' padded post-bag. A set of GBW SUBMISSION SLIPS is included in your survey pack. If you run out of these and need more, please request them from the GBW Team.

The package should be clearly and legibly addressed to the regional laboratory :

The sender's name and address must be clearly written on the back of the package so that they can be contacted in the event of damage or leakage.

The package MUST be labelled in BOLD CAPITALS with the following: 'PATHOLOGICAL SPECIMEN – FRAGILE HANDLE WITH CARE'

Packages must be sent by first class post, guaranteed next day delivery or courier. Parcel post or second class must NOT be used.

Opening times of regional laboratories may vary throughout the week. It is important to avoid parcels arriving at the weekend, when there may not be anyone at the laboratory to receive them, or for parcels to be left in the post for several days. Please check with the laboratory on which day of the week specimens should be sent, dependent on circumstances.

Please let the GBW Team know at the end of each quarter if you would like your postage costs refunded. We will send stamps to the value of the sum requested. Please include a proof of posting form so we can account for the stamps sent to you.

Thank you for your support of the Garden Bird Health initiative. We are very grateful for your involvement in this important project.

If you have any queries please contact the Garden BirdWatch Team on 01842-750050, by emailing gbw@bto.org or by writing to GBHi, GBW Team, BTO, The Nunnery, Thetford, Norfolk, IP24 2PU.

Additional information on this survey appears in the twice-yearly newsletters.

GBHi
THE GARDEN BIRD HEALTH INITIATIVE

Updated 09/2006

BTO/BTO Garden BirdWatch

Garden Bird Health initiative Systematic Surveillance Scheme

INTRODUCTION

Although wild birds are known to be susceptible to a range of diseases, both infectious and non-infectious, we actually know very little about the prevalence of such diseases within populations of birds breeding and wintering within the United Kingdom. Because of the welfare, public health and conservation issues that surround the occurrence of any infectious disease affecting garden birds, there is a need to understand whether there are particular risk factors associated with disease transmission and the potential for outbreaks. For example, we know that the transmission of some diseases between individuals tends to be increased where those individuals gather together in large numbers or where they are physiologically stressed because of other factors. This could suggest that flocks of birds, feeding within a confined area during the winter months, may be at greater risk from certain diseases than they would be at other times of the year.

In order to understand and monitor for disease in wild birds the Garden Bird Health initiative has planned a number of projects, one of which is a systematic surveillance scheme involving 1,000 Garden BirdWatch gardens. Yours is one of the gardens selected for the scheme and we are very grateful to you for agreeing to help with this important project. The following instructions will take you through what is involved and will outline how the various recording forms should be completed. We hope that the instructions are clear and concise but, should you find something that you do not understand, please contact the Garden BirdWatch Team on 01842–750050, by writing to GBHi, GBW Team, BTO, The Nunnery, Thetford, Norfolk, IP24 2PU or by emailing gbw@bto.org.

Where and when to record

Recording for this project follows the format used for Garden BirdWatch, with information on food provision, hygiene practice and signs of disease collected on a weekly basis. Please record within the same area as that used for Garden BirdWatch. For most participants this will be the entire garden but, for some, it may just be part of the garden.

What we would like you to do and why we are asking you to do it

A number of factors may determine the risk of disease transmission at places where birds gather together to feed. We are interested in establishing how the amount and type of food provided, its presentation and the use of particular hygiene measures may influence the occurrence of disease problems. To do this, we need you to keep a more detailed record of the food you provide than we ask for on the standard GBW recording form. How birds feed may also be important – *e.g.* if they feed from a small number of perches or on a small bird table – so we need you to provide information on the types of bird feeders and bird tables in use within your garden. We also need you to look out for evidence of disease in birds visiting your garden and to record any dead birds found. Some of the dead birds found may be useful for post-mortem and you will need to liaise with your nominated Veterinary Centre over which birds to send in and when they should be submitted. Details on how to do this are provided on the last page of these instructions. Contact details for your nominated Veterinary Centre are shown in the box below.

Your nominated Veterinary Centre is:

Becki Lawson MRCVS
Wildlife Epidemiology
Institute of Zoology
Zoological Society of London
regent's Park
London NW1 4RY

Telephone: 0207-449-6685

FORM ONE: THE WEEKLY RECORD FORM

Each of the new weekly record forms covers a GBW quarter. Please return all forms at the end of each quarter. Each form is divided into a number of sections, covering a range of foods, feeder types and hygiene practices. You will need to complete the relevant information for each week in turn and don't forget to include your contact details and GBW number, together with the year and quarter just as you would on your other GBW forms.

Section 1. Weekly Food Provision

Start completing this section by placing a mark in the relevant box to indicate if you carried out recording during the week in question. Then, for each of the food types listed (Sunflower hearts, Black sunflower seed, Mixed seed, Peanuts and Nyjer Seed) we would like you to indicate the quantity or volume of food provided. These should be recorded as 'Small', 'Medium' or 'Large' and should be determined from the following table. Start by either weighing the amount that you put out each time you fill up your feeders or by measuring the volume of food provided. In both cases it is the weekly total that we are interested in. We are interested in the amount of new food put out and not the amount of food available within the feeders. Since weight can be recorded in either ounces or grammes and volume can be recorded as pints or litres, please use the following table to convert your measures to either 'Small', 'Medium' or 'Large'. Hence, 250g of peanuts would be recorded as 'medium', while 200g of black sunflower seed would be 'small'.Once you set up a routine it should be quite easy to keep a record of how much new food you are putting out each week. Please note, we are only interested in the foodstuffs listed in the table below.

		WEIGHT		VOLUME	
		Grammes	Ounces	Litres	Pints
Sunflower hearts	SMALL	less than 300g	less than 10oz	less than 0.5 litre	less than 1 pint
	MEDIUM	300 – 1500g	10 – 50oz	0.5 – 2.5 litre	1 – 4.5 pint
	LARGE	more than 1500g	more than 50oz	more than 2.5 litre	more than 4.5 pint
Black sunflower seed	SMALL	less than 300g	less than 10oz	less than 0.5 litre	less than 1 pint
	MEDIUM	300 – 1500g	10 – 50oz	0.5 – 2.5 litre	1 – 4.5 pint
	LARGE	more than 1500g	more than 50oz	more than 2.5 litre	more than 4.5 pint
Mixed seed	SMALL	less than 300g	less than 10oz	less than 0.5 litre	less than 1 pint
	MEDIUM	300 – 1500g	10 – 50oz	0.5 – 2.5 litre	1 – 4.5 pint
	LARGE	more than 1500g	more than 50oz	more than 2.5 litre	more than 4.5 pint
Peanuts	SMALL	less than 175g	less than 6oz	less than 0.25 litre	less than 0.5 pint
	MEDIUM	175 – 800g	6 – 28oz	0.25 – 1 litre	0.5 – 2 pint
	LARGE	more than 800g	more than 28oz	more than 1 litre	more than 2 pint
Nyjer seed	SMALL	less than 100g	less than 4oz	less than 0.3 litre	less than 0.5 pint
	MEDIUM	100 – 400g	4 – 14oz	0.3 – 1 litre	0.5 – 2 pint
	LARGE	more than 400g	more than 14oz	more than 1 litre	more than 2 pint

Section 2. Seed and Peanut Feeders

Section 2a. asks for information on two common types of hanging or pole-mounted feeders (MESH PEANUT FEEDERS and PLASTIC TUBE SEED FEEDERS). Please start by recording the number of Mesh Peanut Feeders you had in use within your garden, using the categories shown ('0','1','2' or '3+'). Then note if you cleaned ANY of these feeders during the week. Then record the number of Plastic Tube Seed Feeders in use within your garden, again using the categories shown ('0','1','2' or '3+'). Next, record the the number of feeding ports available on these feeders by using the categories ('0','1–8','9–19' or '20+). All ports at which a bird can perch, regardless of whether the level of available food reaches the port or not, should be recorded. Finally, note if you cleaned ANY of these tube feeders during the week. *Section 2b.* is used to collect information on any hygiene measures used on these two types of feeder. For each week, please note if you used any of the following cleaning products ('just water','detergent' or 'disinfectant') to clean ANY of the Mesh Peanut Feeders or Plastic Tube Seed Feeders recorded in Section 2a. The use of cleaning products on other feeders or bird tables should be recorded in Section 3b.

Section 3. Trough Feeders, Bird Tables, etc.

Section 3a. asks for information on a range of other common ways of providing food (and water) for garden birds. For each week, please start by recording the number of TROUGH FEEDERS you had in use within your garden, using the categories shown ('0','1','2' or '3+'). Then note if you cleaned ANY of these feeders during the week. A trough feeder has a small tray at its base and a food hopper above, from which food moves into the tray (see photograph at foot of page). Repeat this process for TRAY FEEDERS, COVERED BIRD TABLES and UNCOVERED BIRD TABLES. A covered bird table is one that has a roof, while an uncovered does not have a roof.

Next, record if you INTENTIONALLY provided any food on the ground by placing a mark in the appropriate box, before going on to note whether or not you cleaned the ground where you provided the food. Cleaning the ground is taken to include: sweeping the area clean, hosing down the area or using a powder-based detergent product (e.g. Garden Klens) sprinkled onto the area.

Finally, please mark the appropriate box to show how many BIRD BATHS you had in use within your garden. Please only record bird baths and NOT ponds or other natural sources of water. For each week, please record if you cleaned ANY of you bird baths.

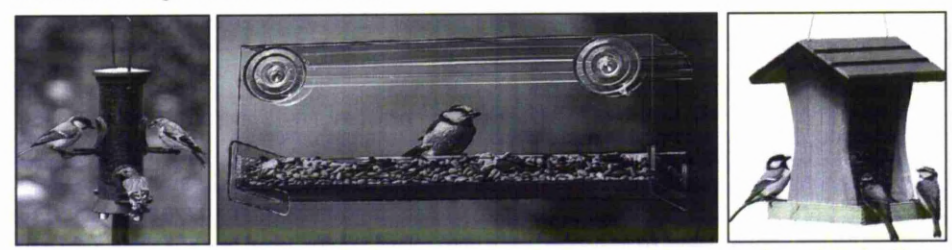
Section 3b. is used to collect information on any hygiene measures used on these other common ways of feeding and watering garden birds. For each week, please note if you used any of the following cleaning products ('just water','detergent' or 'disinfectant') to clean ANY of the devices recorded in Section 3a.

Section 4. Signs of disease and dead birds found

During the course of each week, we would like you to keep an eye out for birds showing signs of disease (*e.g.* appearing lethargic, fluffed up or unwilling to move away from feeding stations). Other birds may be found dead within the garden. Please take a regular walk around your garden, at least once each week, checking under bushes to help you find dead birds. We would like you to record **all** birds found dead in the garden, no matter how they died, by completing *Section 4.* AND FORM TWO. Please place a mark in the appropriate box if you saw any evidence of disease in the birds in your garden. This can include birds looking fluffed up, lethargic or with growths on their legs. Do the same for the question which asks if you found any dead birds. If you find a dead bird or see any signs of disease, please enter the details on FORM TWO. Remember to include the date on which the bird was found/seen, the species (if known) and the suspected cause of death or illness (if known). Full instructions on completing FORM TWO are included on the form itself.

What to do if you find a dead bird

If you find a dead bird in the garden, you need to call your designated regional laboratory and make arrangements for submission before the specimen is packaged or sent. In this way, we can check whether the specimen is suitable for examination. Only freshly dead carcasses are suitable for post-mortem examination. If you are unsure whether the garden bird is in a suitable condition, please discuss this with the regional laboratory during your call. Specimens may deteriorate rapidly and so it is important to contact the regional laboratory as soon as possible after finding a specimen. It is likely that not all birds for which the suspected cause of death is known (*e.g.* window strikes or cat predation) will need to be sent off for post-mortem. However, valuable information regarding disease occurrence within the wider bird population can be gained from post-mortem of these birds. Since resources are limited, the decision as to whether to examine a particular bird will be made on a case by case basis, something which your regional laboratory will discuss with you. Remember to mention that you are a BTO/CJ Garden BirdWatcher participating in the Systematic Surveillance Scheme to distinguish you from calls made by members of the public. Please follow the guidelines shown overleaf.



From left: A plastic tube seed feeder, with four feeding ports; a tray feeder; a trough feeder, complete with the food hopper that distinguishes this from a simple tray feeder. Images supplied by CJ WildBird Foods Ltd.

Garden Bird Health Initiative
Systematic Surveillance Scheme
WEEKLY RECORD FORM

PLEASE ENTER YOUR NAME, GBW NO., POSTCODE AND WEEK

Name: _____ YEAR: 2006

GBW Number: _____ GBW Quarter: 1

POSTCODE: _____ GBW Week Number: _____

1 Did you undertake observations for this survey during this week? ('Yes'/'No')

2 Food provision

For each of the food types shown in the table please record if it was provided this week and, if so, how much was put out. The presence of other food types is recorded on your normal GBW form. You can express the amount provided either by weight or by volume. Use ounces or grammes for weight and pints or litres for volume. Please remember to note the units used.

Food type	Provided this week (Y/N)	Amount by weight		Amount by volume	
		Weight of food provided (ounces or grammes).	oz/g	Volume of food provided (pints or litres).	P/L
Sunflower hearts					
Black sunflower seed					
Mixed seed					
Peanuts					
Nyjer seed					

3 Feeders and bird tables (see instructions for descriptions of types)

For each type of feeder, bird table or other feeding location, please note the number in use during the week, the number of perches/ports or table area and the cleaning regime used.

	Number provided by you this week	Total number of ports or perches available (see instructions)	Total area of bird table (cm^2 or $inches^2$) (see instructions)	How many tables/tray feeders were brushed down this week (see instructions)	How many were cleaned this week (see instructions)	What cleaning agents were used? Include water, any detergents, specialist cleaning agents like 'ark-klens' and any special disinfectant powder for ground use ('garden-klens'). If in doubt, please contact the GBW Team.
Mesh peanut feeder						
Plastic tube seed feeder						
Trough feeder						
Tray feeder						
Covered bird table						
Uncovered bird table						
Feeding on the ground						
Bird baths (not ponds)						

4 Signs of disease and dead birds recorded

Please tell us if you saw any evidence of diseased birds this week. Please enter details of any dead birds found. See instructions for more details.

Did you see any signs of diseased birds this week (Yes or No)?

Did you find any dead birds this week (Yes or No)? If so, please enter their details below.

Date	Species of bird (if known)	Suspected cause of death if known (see instructions)	Sent for post-mortem (Y/N)	Identification number if sent for post-mortem

WILDBIRD FOODS LTD
the wild bird specialists

Garden Bird Health Initiative

BTO

PLEASE REFER TO YOUR INSTRUCTION SHEET FOR GUIDANCE ON HOW TO FILL IN THIS FORM
Mark all the appropriate boxes in pencil before checking them over – in case of mistakes.
Then fill in the appropriate boxes completely like this ▬, using a black or blue pen only (NOT felt-tip).

NAME AND ADDRESS

POSTCODE

Year/Quarter

Q1	2006
Q2	2007
Q3	2008
Q4	2009

For Garden BirdWatchers only: YOUR GBW NUMBER

Please fill in the five digits of your Garden BirdWatch number. *(Mark one box per line).*

1st digit	0	1	2	3	4	5	6	7	8	9
2nd digit	0	1	2	3	4	5	6	7	8	9
3rd digit	0	1	2	3	4	5	6	7	8	9
4th digit	0	1	2	3	4	5	6	7	8	9
5th digit	0	1	2	3	4	5	6	7	8	9

SECTION 1. WEEKLY FOOD PROVISION

WEEK NUMBER

		1	2	3	4	5	6	7	8	9	10	11	12	13
lace a mark in the box to indicate that you carried out recording during this week														
or each week in turn, please mark the appropriate box if you provided SUNFLOWER HEARTS														
lease mark 'small', 'medium' or 'large' to record the quantity provided. ee the printed instruction sheet to establish the quantity or volume of this ood type associated with each category.	Small													
	Medium													
	Large													
or each week in turn, please mark the appropriate box if you provided BLACK SUNFLOWER SEED														
lease mark 'small', 'medium' or 'large' to record the quantity provided. ee the printed instruction sheet to establish the quantity or volume of this ood type associated with each category.	Small													
	Medium													
	Large													
or each week in turn, please mark the appropriate box if you provided MIXED SEED														
lease mark 'small', 'medium' or 'large' to record the quantity provided. ee the printed instruction sheet to establish the quantity or volume of this ood type associated with each category.	Small													
	Medium													
	Large													
or each week in turn, please mark the appropriate box if you provided PEANUTS														
lease mark 'small', 'medium' or 'large' to record the quantity provided. ee the printed instruction sheet to establish the quantity or volume of this ood type associated with each category.	Small													
	Medium													
	Large													
or each week in turn, please mark the appropriate box if you provided NYJER SEED														
lease mark 'small', 'medium' or 'large' to record the quantity provided. ee the printed instruction sheet to establish the quantity or volume of this ood type associated with each category.	Small													
	Medium													
	Large													

SECTION 2a. SEED AND PEANUT FEEDERS – provision and cleaning

WEEK NUMBER

		1	2	3	4	5	6	7	8	9	10	11	12	13
or each week in turn, please mark the appropriate box to show how many MESH PEANUT FEEDERS you had in use within your garden.	0													
	1													
	2													
	3+													
or each week, please note if you cleaned any of your MESH PEANUT FEEDERS.	Yes													
	No													
or each week in turn, please mark the appropriate box to show how many LASTIC TUBE SEED feeders you had in use within your garden.	0													
	1													
	2													
	3+													
or each week in turn, please mark the appropriate box to show how many orts were available, in total, on your PLASTIC TUBE SEED FEEDERS.	0													
	1-8													
	9-19													
	20+													
or each week, please note if you cleaned any of your plastic tube seed feeders.	Yes													
	No													

SECTION 2b. SEED AND PEANUT FEEDERS – cleaning agents used

or each week in turn, please mark the appropriate box to show if you sed any of the following cleaning agents to clean either your mesh eanut feeders or your plastic tube seed feeders. Cleaning agents used o clean other feeder types or bird tables should be noted overleaf. SEE instruction sheet for more information).	just water													
	detergent													
	disinfectant													

Please turn over...

SECTION 3a. TROUGH FEEDERS, BIRD TABLES, ETC. – provision and cleaning

		WEEK NUMBER												
		1	2	3	4	5	6	7	8	9	10	11	12	13
For each week in turn, please mark the appropriate box to show how many TROUGH FEEDERS you had in use within your garden.	0													
	1													
	2													
	3+													
For each week, please note if you cleaned any of your TROUGH FEEDERS.	Yes													
	No													
For each week in turn, please mark the appropriate box to show how many TRAY FEEDERS you had in use within your garden.	0													
	1													
	2													
	3+													
For each week, please note if you cleaned any of your TRAY FEEDERS.	Yes													
	No													
For each week in turn, please mark the appropriate box to show how many COVERED BIRD TABLES you had in use within your garden.	0													
	1													
	2													
	3+													
For each week, please note if you cleaned any of your COVERED BIRD TABLES.	Yes													
	No													
For each week in turn, please mark the appropriate box to show how many UNCOVERED BIRD TABLES you had in use within your garden.	0													
	1													
	2													
	3+													
For each week, please note if you cleaned any of your UNCOVERED BIRD TABLES.	Yes													
	No													

FOLD HERE

		WEEK NUMBER												
		1	2	3	4	5	6	7	8	9	10	11	12	13
For each week in turn, please mark the appropriate box to show IF YOU PROVIDED FOOD ON THE GROUND INTENTIONALLY.														
For each week in turn, please mark the appropriate box to show IF YOU CLEANED THE AREA WHERE YOU PROVIDED FOOD ON THE GROUND.	Yes													
	No													
For each week in turn, please mark the appropriate box to show how many BIRD BATHS you had in use within your garden.	0													
	1													
	2													
	3+													
For each week, please note if you cleaned any of your BIRD BATHS.	Yes													
	No													

SECTION 3b. TROUGH FEEDERS, BIRD TABLES, ETC. – cleaning agents used

		WEEK NUMBER												
		1	2	3	4	5	6	7	8	9	10	11	12	13
For each week in turn, please mark the appropriate box to show if you used any of the following cleaning agents to clean any of the feeders/bird baths, etc. shown on THIS SIDE of the recording form. Cleaning agents used to clean other mesh peanut feeders or plastic tube seed feeders should be noted overleaf. (SEE instruction sheet).	just water													
	detergent													
	disinfectant													

SECTION 4. SIGNS OF DISEASE AND DEAD BIRDS FOUND

	WEEK NUMBER												
	1	2	3	4	5	6	7	8	9	10	11	12	13
For each week, please place a mark in the appropriate box if you saw any evidence of disease in the birds in your garden, for example birds looking fluffed up, lethargic or other signs.													
For each week, please place a mark in the appropriate box to note if you found any dead birds in your garden.													

PLEASE COMPLETE THE SEPARATE 'DISEASED AND DEAD BIRD RECORDING FORM' WITH ANY RECORDS OF BIRDS SHOWING SIGNS OF DISEASE OR FOUND DEAD WITHIN YOUR GARDEN. PLEASE RETURN COMPLETED FORMS AT THE END OF THE QUARTER TO GBHI, GBW , BTO, THE NUNNERY, THETFORD, NORFOLK, IP24 2PU.

GBH*i* POST MORTEM EXAMINATION

Common name:
Scientific name:

PME Number (IOZ/LIV/SAC/WVIC - PM No (four digits) -Year):
AM Number (IOZ/LIV/SAC/WVIC - AM No (four digits) -Year):
In house PME Number:
Report type (Opportunist or Systematic)?
GBW Number (GBW site id – Bird number):
PM Date:
Pathologist: Becki Lawson

Name, Address & Tel. No of Finder:

NGR (6 digits):

Date found:
Date received:
Summary history:

Euthanased?

Carcass state (Fresh/ Frozen):
Carcass condition (Freshly dead/ mild autolysis/ moderate decomposition/ advanced decomposition):
Sex (Male/ Female/ Undetermined):
Age (Nestling/ Juvenile/ First year (of life)/ Adult):
Body condition (Emaciated/ Thin/ Normal/ Fat):
BWG Fat score (0-8):
Carcass weight (g):
Wing length (mm):
Maximum tarsus length (mm):
Photos(s) taken (Y/N)?
Xray(s) taken (Y/N)?
Leg ring present (Y/N)?

Gross post mortem findings

Integument (plumage, moult, uropygial gland, subcut.)		
Sensory (eyes, ears, nostrils)		
Muscular		Total Sup. Pect. Mass (g)
Skeletal		
Cavities		
Digestive		
Liver		(g)
Respiratory		Lungs NA
Cardio-vascular		Heart NA
Lympho-reticular (Spleen, Bursa, Thymus)		Spleen NA
Urinary		Total Kidney NA
Endocrine (Thyroid, Adrenal)		
Reproductive		
Nervous		

Key:
NA Not available
NE Not examined
NLD No lesions detected
Values in parentheses not reliable.

PARASITOLOGY
Small intestinal contents wet smear:

MICROBOLOGY
Liver:
SI:

MISCELLANEOUS

HISTOLOGY
Tissues stored in 10% BFS archive include:

DIAGNOSIS

Significant diseases or conditions thought to contribute to the death of the animal

Incidental diseases or conditions not thought to contribute to the death or condition causing it

Comments:

CAUSE OF DEATH:

Trauma / Predation (Avian/ Mammal/ Undetermined)/ Infectious disease (Pathogen)/ Other/ Undetermined

**This report is based on gross findings and may be modified after the laboratory findings are known. (*Delete when PME report complete).*

Garden Bird Health Initiative Systematic Surveillance Scheme Spring 2007 Update

Thank you for your continued support of the Garden Bird Health initiative (GBHi). The project is just about to enter its seventh quarter of recording (of 12 planned quarters) and continues to produce much-needed information on disease risks faced by garden birds. It is clear that the new design of recording form is working much better than the old one and we are very grateful for all the comments received that helped us shape the new design of form.

What happens to your birds?

A number of participants have asked what happens to the birds that are sent off for post mortem examination (PME), so we asked Becki Lawson from the Institute of Zoology to explain.

Birds that arrive at the designated laboratories are stored in the refrigerator on arrival and are examined as soon as possible, preferably on the same day. Many of the tests that we run are best performed on fresh samples and so, by prioritising this work, we can get the maximum information from the study. Details on the species, age and sex of the bird are recorded as routine. Body weight and other standard biometric measurements are taken from each case. General body condition is assessed to see if the bird is in a good or poor nutritive status. Photographs are taken of abnormalities or other observations of interest.

Following a systematic protocol, body organs are examined in sequence and abnormalities are noted. Visible parasites are archived in a chemical fixative for species identification and the contents of the digestive system are routinely screened for parasite eggs under the microscope. Samples are then given to our microbiologists who culture them to identify any significant bacteria or fungi present. This process typically takes a period of up to five days, dependent on findings. In some instances, unusual isolates are submitted to specialist national laboratories for further characterization.

A suite of samples is taken from each bird, with tissues preserved in fixative, for processing and examination under the microscope, and in the freezer. This archive is a unique and valuable resource which will enable us to do studies in the future, focusing on particular species of bird or specific diseases. The post mortem examination typically takes an hour to perform and other test results are available after around a week. At this stage the findings are reviewed and a category is assigned for the cause of death; for example trauma, predation or infectious disease. In some instances, additional tests may be required at external specialist laboratories to help reach a diagnosis; in other cases it may not be possible to conclude with certainty the reason why the bird died.

All of the diagnostic laboratories that take part in the GBH*i* use similar examination protocols to allow their results to be collated. Findings from across Britain are reviewed each year, *e.g.* the seasonal and geographical distribution of a particular disease in garden birds can be monitored through the findings of the Garden BirdWatch volunteer network.

Results from the various laboratories, once collated, are passed to the BTO, where results for individual birds are matched with the other information submitted on the weekly recording forms. At this stage, a copy of the findings can be sent to each participant who submitted a bird. Some of the veterinary laboratories provide separate feedback earlier than this, but the mailing of standard information annually from the BTO also helps to check that the data held on the database are correct. The next batch of post mortem results should be available within the next few weeks and we will send this out to those participants who have submitted carcasses over the last few quarters.

Chaffinch with leg wart
BTO Collection

Warts and other growths on the legs of birds are caused by a number of different agents. We would like to quantify the prevalence of these different agents in our bird populations. To help us do this, PLEASE SEND ANY DEAD BIRDS SHOWING LEG GROWTHS TO YOUR DESIGNATED VETERINARY LABORATORY. Thank you!

Disease over the winter

Feedback from the veterinary laboratories has highlighted an interesting pattern to mortality events reported over the autumn and winter months. With fewer birds in gardens this winter, thanks to the abundance of wild food and mild weather conditions, it is unsurprising that outbreaks of the typical "winter" diseases have been few and far between. Reports of birds with Salmonellosis tend to be most pronounced during the second part of the winter and there have been relatively few cases reported this year.

One of the most interesting patterns to have emerged over the last two quarters is the continued occurrence of birds suffering from trichomoniasis. Readers will recall that this disease was first identified in finches (notably Greenfinches) in late summer 2005. A much bigger outbreak occurred in late summer 2006, being centred on the southwest of England, the West Midlands and northwest England. Although, as in 2005, the number of reports dropped off during late autumn, we have had continued cases of this disease reported through the winter months. Again, it is the Greenfinch population that has been the worst hit, but other finch species and House Sparrow have also been affected. Having a systematic monitoring scheme in place, through the Garden Bird Health initiative, provides a wonderful opportunity to follow the progress of such outbreaks, highlighting the regions and species that are involved.

Contacting the veterinary labs

Please do continue to report any dead birds found to your designated laboratory, regardless of whether or not you suspect that disease may be involved. It is extremely important that we carry out post mortem examinations of birds suspected of dying from a range of different causes, including window strikes and predation. By doing so, we can examine the background levels of diseases, parasites and other pathogens, and explore how these may make birds more susceptible to predation, *etc.*

Siskins were scarce at garden feeding stations this winter, thanks to abundant food in the wider countryside. Photograph by John Harding.

Bird species identified by participants, suspected cause of mortality and numbers sent for post mortem examination (PME). DI = Disease suspected, PR = Predation suspected, TR = Trauma suspected and UN = Unknown cause. PME = number submitted. (2005 Q4 to 2006 Q4 inclusive).

SPECIES	No.	DI	PR	TR	UN	PME
Greenfinch	591	117	136	175	163	109
Chaffinch	293	70	60	93	70	74
Blackbird	248	4	93	103	48	37
House Sparrow	201	12	93	49	47	26
Collared Dove	145	12	83	21	29	15
Blue Tit	110	0	53	41	16	13
Woodpigeon	87	4	48	18	17	10
Goldfinch	78	6	26	31	15	14
Dunnock	67	0	30	22	15	12
Great Tit	61	0	23	34	4	10
Siskin	61	9	4	20	28	27
Robin	54	1	30	14	9	9
Starling	44	0	18	18	8	6
Feral Pigeon	41	1	26	4	10	3
Song Thrush	24	0	2	19	3	6
Great Sp. W.pecker	19	0	3	11	5	6
Tree Sparrow	15	1	11	3	0	2
Bullfinch	14	0	2	11	1	4
Coal Tit	14	0	4	7	3	5
Blackcap	12	0	0	10	2	4
Wren	11	0	6	2	3	-
Jackdaw	8	1	2	3	2	2
Long-tailed Tit	8	0	2	5	1	4
Goldcrest	7	0	0	7	0	4
Swallow	7	0	2	4	1	1
Magpie	6	0	4	1	1	1
Pheasant	6	0	4	1	1	-
Unknown	103	1	74	5	23	3

Records were received for a further 33 species, including: Redwing, Sparrowhawk, Nuthatch, Brambling, Moorhen, Redstart, Ring-necked Parakeet, Treecreeper & Jay.

Useful Contacts

Garden BirdWatch, BTO, The Nunnery, Thetford, IP24 2PU, 01842–750050 or gbw@bto.org for all general enquiries about the project.

The four participating veterinary laboratories are listed below. Please contact your designated centre (see your original instructions).

Becki Lawson MRCVS, Wildlife Epidemiology, Institute of Zoology, Zoological Society of London, Regent's Park, London, NW1 4RY. 0207–449–6685.

Dr Julian Chantrey MRCVS, Dept. of Veterinary Pathology, Liverpool University, Leahurst Veterinary Teaching Hospital, Chester High Road, Neston, Wirral, CH64 7TE. 0151–794–6012.

Tom Pennycott MRCVS, SAC Veterinary Services, Avian Health Unit, Auchincruive, Ayr, KA6 5AE. 01292–520318.

Mr Vic Simpson Hon FRCVS, Wildlife Veterinary Investigation Centre, Jollys Bottom Farm, Station Road, Chacewater, Truro, Cornwall, TR4 8PB. 01872–560623

Disease and garden birds

iseases may be passed from one ndividual to another through food that as become contaminated with faeces. his suggests that transmission rates are kely to be lower where birds feed on anging feeders with a narrow perch, an where they feed on the ground or n a bird table. Good hygiene practice ee leaflet), like sweeping down your ird table each day, should reduce ansmission risk. Greenfinch on hanging eeder by Mike Toms.

Outbreaks of disease may occur in populations of wild birds wherever they occur, including those visiting gardens. Because garden birdwatchers take so much interest in the birds visiting their gardens, they may occasionally come across diseased birds and a number of different diseases have been reported from birds seen at garden feeding stations. These include those caused by *Salmonella* and *E. coli* but other, less familiar, diseases may also occur. It is well-known that the transmission of disease between individuals tends to be increased where animals or birds gather together in large numbers and this may go some way to explaining why some species (*e.g.* Greenfinch, House Sparrow, Siskin, Collared Dove and Chaffinch) may be more susceptible than others. Worldwide, there are occasional reports of large-scale outbreaks of diseases like Salmonellosis in small birds, some of which are linked to garden feeding stations. Of course, such outbreaks may also occur in other habitats where they would largely go unnoticed.

The occurrence of infectious diseases affecting garden bird species raises a number of welfare, conservation and, potentially, public health issues. As a consequence there is a clear need to understand the prevalence of these diseases, the species affected and what risk factors might be associated with infection. For example, do the volume of food provided, the pattern of hygiene measures employed, time of year and/or weather conditions influence the chances of infection? Research aimed at answering such questions has been largely opportunistic and there has been no nationally-based systematic monitoring scheme in place. Until now that is.

This summer saw the launch of the Garden Bird Health initiative, an idea that arose from a Universities Federation for Animal Welfare (UFAW) working group set up to identify and address the need for disease surveillance and research. BTO staff have been working alongside representatives from UFAW, The University of Liverpool Veterinary School, Institute of Zoology, RSPB, Jacobi Jayne & Co, Cranswick Pet products, Gardman Ltd, the Scottish Agricultural College, Defra and CJ WildBird Foods Ltd to set up a disease surveillance project that will begin this autumn. Funding for the project has come from a number of sources, with the bulk of it coming from the collaborating organisations and, in particular, those representing the bird feeding industry. We are very grateful for the tremendous amount of support that they have given to this project.

Central to the collection of information for this project is a network of 1,000 BTO/CJ Garden BirdWatchers who have been selected to provide an unbiased sample of garden types and hygiene practices from across Britain. In addition to their weekly reporting of bird observations, these volunteers will also keep more detailed records of the food they provide, the hygiene practices they follow and any evidence of birds showing signs of disease. This will include a systematic search of their garden each week to look for dead birds. These will be collected and sent off for post-mortem at a series of veterinary laboratories organised on a regional level. The results of the post-mortems will be collated in a centralised database, so that they can be analysed in a way that should reveal answers to the questions outlined earlier in this article.

Over the next couple of years we will be able to report back on the findings of the work, through articles in ***Bird Table***, and we hope to be able to refine our advice on best practice for maintaining hygiene levels at garden feeding stations. In order to get the ball rolling, so to speak, we have prepared a new leaflet on Hygiene and Disease and we are delighted to be able to include a copy in with this mailing. Further copies are available by contacting the Garden BirdWatch Team at the usual address. If you are not one of those selected for involvement in the project, you may be interested to know that should you encounter a disease outbreak, you can still contribute to the project by submitting carcasses for post-mortem. More details on this appear in the leaflet in the box titled 'Garden Bird Health initiative'. Alternatively, you can telephone 0207–449–6685. A new section on Hygiene and Disease will appear on the Garden BirdWatch website (www.bto.org/gbw) during September.

Mike Toms

Garden Bird Health initiative

op: Greenfinch (John Harding) is the pecies for which we have received ıe most *Trichomonas*-related reports ıis summer. Below: Participants in ıe systematic surveillance scheme ıre recording both food provision ınd hygiene practice alongside their egular Garden BirdWatch counts. ome feeders are not as clean as ıey should be. Goldfinch by Jill akenham.

As many readers will be aware, BTO/CJ Garden BirdWatchers are at the heart of a new project examining the prevalence of disease in garden birds. This project is part of the ***Garden Bird Health initiative***, a collaboration between a number of organisations and companies all concerned about the welfare of garden birds (see box, bottom left).

Over the course of the last fifteen months, nearly 1,000 Garden BirdWatch volunteers have been collecting very detailed information on the ways in which they are presenting food for visiting birds and managing their hygiene routines. In addition, they have been carrying out regular checks looking for evidence of diseased or dead birds within their gardens. Many of the dead birds found are then sent off for post-mortem examination (PME) at participating veterinary laboratories around the country. This article outlines some of the initial findings and also brings you up to date on the diseases seen during 2006, including – most notably – the outbreak of trichomoniasis.

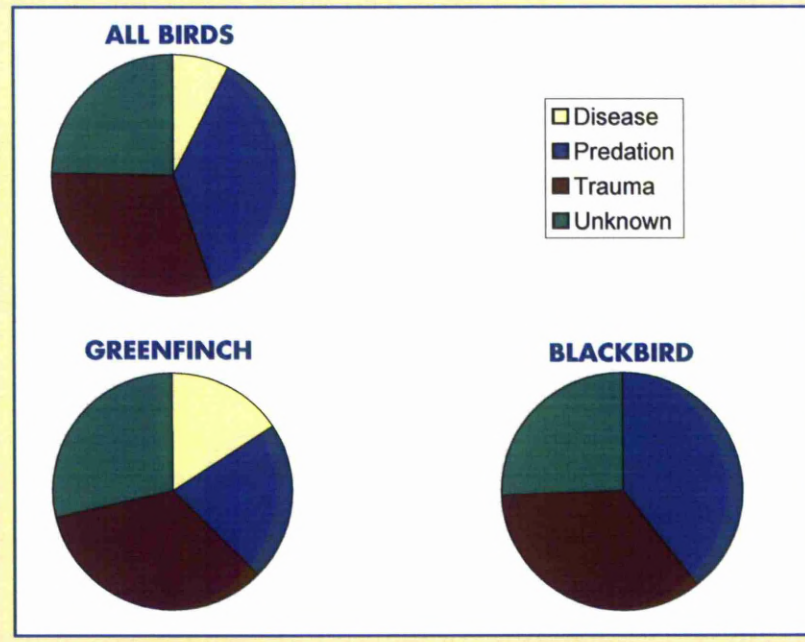
Mortality causes identified through GBHi

During the first six months of the project (October 2005–March 2006), Garden BirdWatchers participating in the systematic surveillance scheme reported some 838 mortality incidents. These involved 37 different species of bird and most were thought to be the result of predation, typically by a Cat or Sparrowhawk, or trauma, often involving windows (see box below).

Each participant has been given a nominated veterinary laboratory to which some of the dead birds found are sent. The post-mortem examinations (PME) so far carried out have documented the presence of diseases such as salmonellosis, trichomoniasis (see box on Page 11) and yersinosis. By examining the occurrence of these diseases in relation to the location, feeding regime and hygiene practice at sites we hope to build up an understanding of disease risks and the factors that may favour disease transmission between individual birds. We can also use the bird

Suspected mortality causes for (a) all birds, (b) Greenfinch and (c) Blackbird

The pie charts show the breakdown of suspected mortality causes as recorded by participants prior to birds being sent off for post-mortem examination. We will be able to compare suspected mortality causes with actual cause of death by looking at the results of the post-mortem examinations.



GBHi contributors

The GBHi is a collaboration between the following organisations:

BirdCare Standards Association
British Trust for Ornithology
British Veterinary Association Animal Welfare Fund
CJ WildBird Foods Ltd
Cranswick Pet Products
University of Liverpool Veterinary School
Gardman Ltd
Institute of Zoology
Royal Society for the Protection of Birds
Scottish Agricultural College
Health Protection Agency
Universities Federation for Animal Welfare
Wildlife Veterinary Investigation Centre

d Table 48 page 10

count information from the weekly Garden BirdWatch recording to examine the extent to which the numbers of birds using a feeding station may influence disease risk. We also hope that PMEs of birds known to have died from trauma (*e.g.* by flying into a window) will reveal information on the background levels of diseases, like salmonellosis, in the wild bird population. We know, for example, that some birds are positive (on PME) for both *Salmonella* and *Trichomonas*.

Other developments

There is a great deal of other work going on through the Garden Bird Health initiative. For example, staff at the Institute of Zoology are working on molecular characterisation of the *Trichomonas* parasite. By doing this they hope to find out if the *Trichomonas* seen last summer in finches and sparrows is a new strain or if it originated from another source, such as pigeons. Knowing this will help determine the routes of disease transmission and should help to strengthen recommendations about best feeding practice.

Through the Garden Bird Health initiative, the collaborating organisations and companies also hope to continue developing guidelines about how best to feed garden birds, whilst minimising disease and other risks. Central to this will be the scientific and research work based around the systematic surveillance scheme. We have just started on our second year of what is, initially, a three-year study. As we progress, we will begin to unravel the interactions between disease occurrence and transmission, and the range of factors that have been suggested may influence the likelihood of birds passing disease from one to another.

Mike Toms

Latest news

The veterinary laboratories participating in the Garden Bird Health initiative have just reported their first cases of salmonellosis of the winter. The symptoms are similar to those of trichomoniasis, with birds appearing fluffed-up, lethargic and unwilling to move away from feeding stations. Unlike trichomoniasis, salmonellosis can be passed to humans and domestic animals (*e.g.* pet cats). Again, good hygiene practice is essential to reduce the chances of the disease being transmitted between birds.

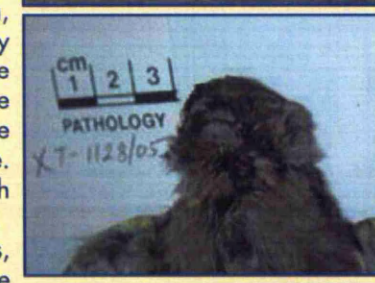
Trichomoniasis

Trichomoniasis is the name given to a disease caused by the single-celled parasite *Trichomonas gallinae*, one of a number of related organisms known as Trichomonads. The disease was historically associated with pigeons and doves, and the birds of prey that fed on them. Some readers will be familiar with other names for the disease, notably "canker" and "frounce". Since summer 2005 the disease has also been seen in finches, with a significant outbreak noted during summer 2006 attracting widespread media attention.

Birds suffering from the disease show signs typical of general illness, namely appearing lethargic, fluffed-up and reluctant to leave feeding stations. In addition, affected birds may have difficulty in swallowing, drool saliva or regurgitate food. Many of the reports received this year refer to finches with wet or matted plumage around the beak and face. Because the disease causes lesions at the back of the throat and in the gullet, affected individuals are unable to swallow food and become emaciated. The disease seems to progress over several days, or even weeks, before the individuals die. During this period they may pass the infection onto other individuals, either through food or water contaminated with recently regurgitated saliva or, possibly, droppings.

While medicines are available for the treatment of the disease in captive birds, effective dosing of wild birds is not possible. As such, prevention is the most appropriate measure for controlling the disease. When feeding garden birds maintain good hygiene practice. For example: clean feeders regularly (weekly or even daily), provide fresh water on a daily basis and rotate feeding locations around the garden to prevent the build up of contamination in any one area.

Where a problem with trichomoniasis occurs, with large numbers of birds seen sick or dying, make a real effort to thoroughly clean all feeding and watering equipment, with disinfectant (refer to either the GBHi booklet or the GBW leaflet for more detail on this) and, if problems continue, consider suspending feeding for a short period while diseased birds are present within the garden. Remember to wear rubber gloves while cleaning feeders and thoroughly wash hands and forearms afterwards with soap and water. Avoid handling sick or dead birds directly. **For more advice visit the following web pages or call the GBW Team: www.bto.org/gbw/NEWS/disease_outbreak.htm or http://www.ufaw.org.uk/gbhi.php.**

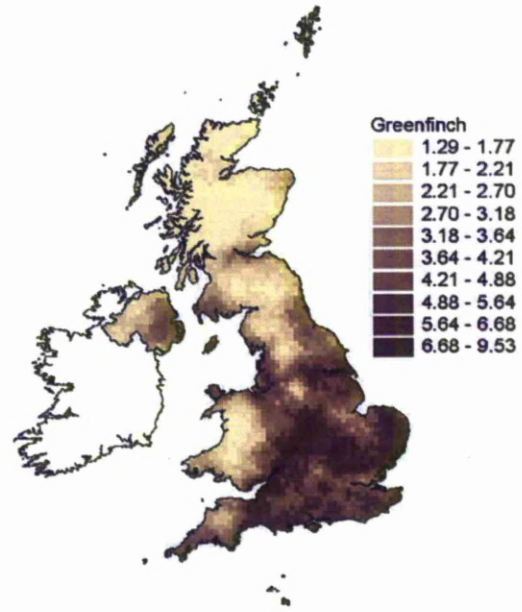


From top: House Sparrow by John Harding. Middle: Greenfinch with matted plumage around face and vent (both by Becki Lawson, Institute of Zoology).

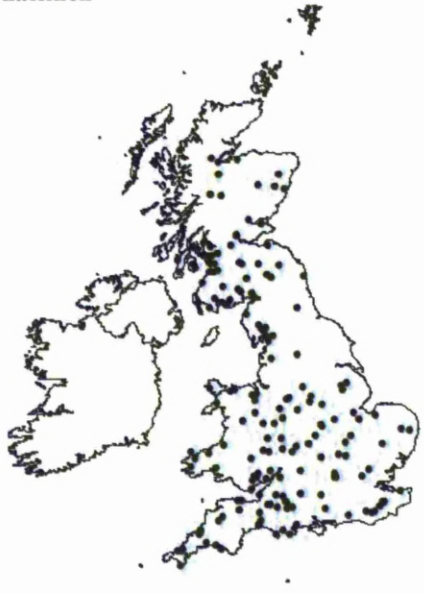
Appendix 8 Geographical distribution of species submitted for PME (1st April 2005 – 31st March 2008)

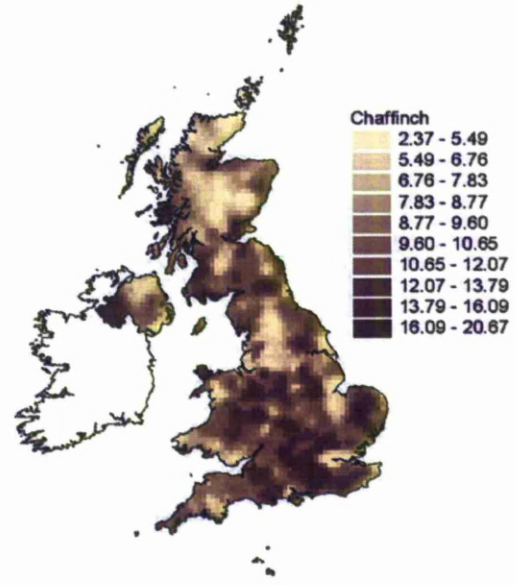
Species displayed in rank order on basis of number of submissions, together with a map showing relative abundance based on Breeding Bird Survey data from 2003. The Breeding Bird Survey (BBS) monitors terrestrial birds throughout the UK to provide information that underpins the conservation of species and habitats. The BBS is organised by the BTO on behalf of BTO, JNCC and RSPB. Comparison of the distribution of submissions and the BBS data enables us to evaluate how comprehensive the surveillance that we have been able to achieve for each species has been.

a) Greenfinch

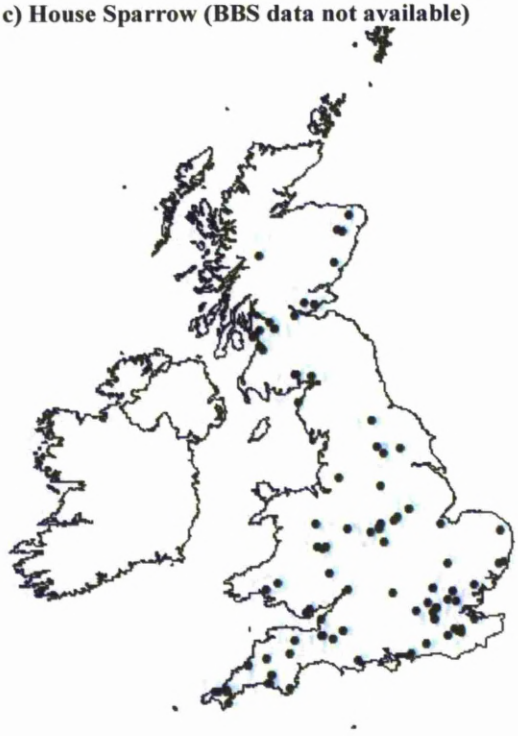


b) Chaffinch

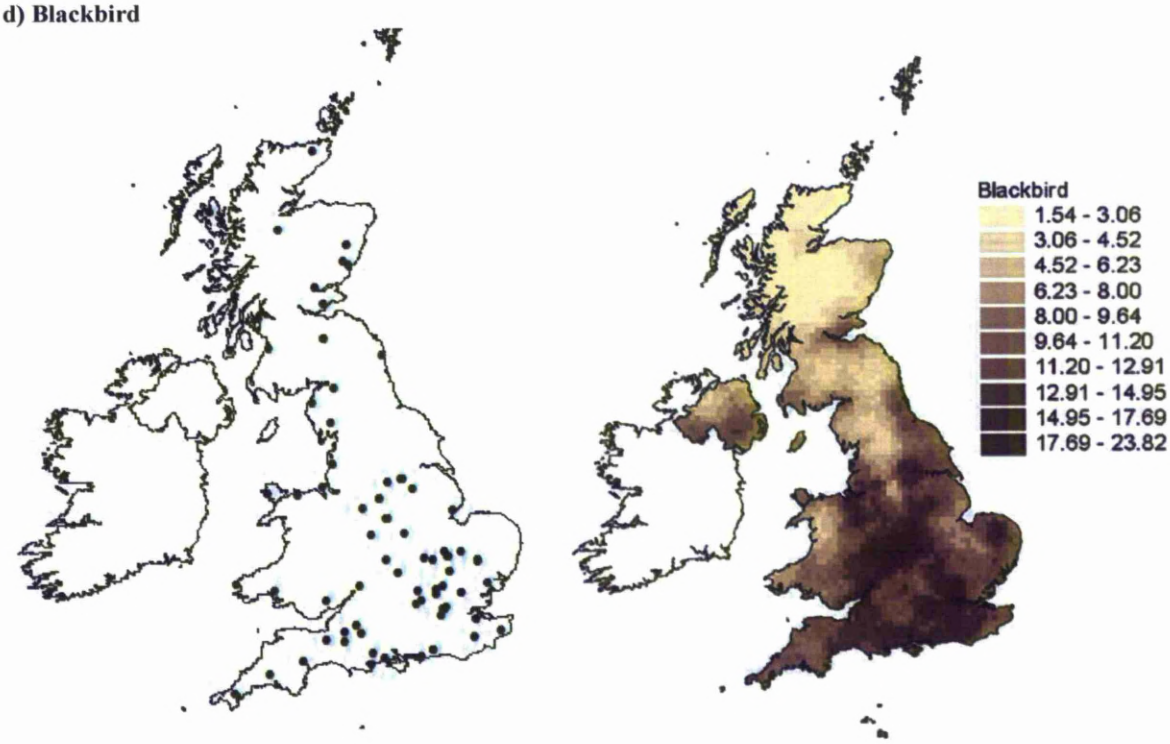




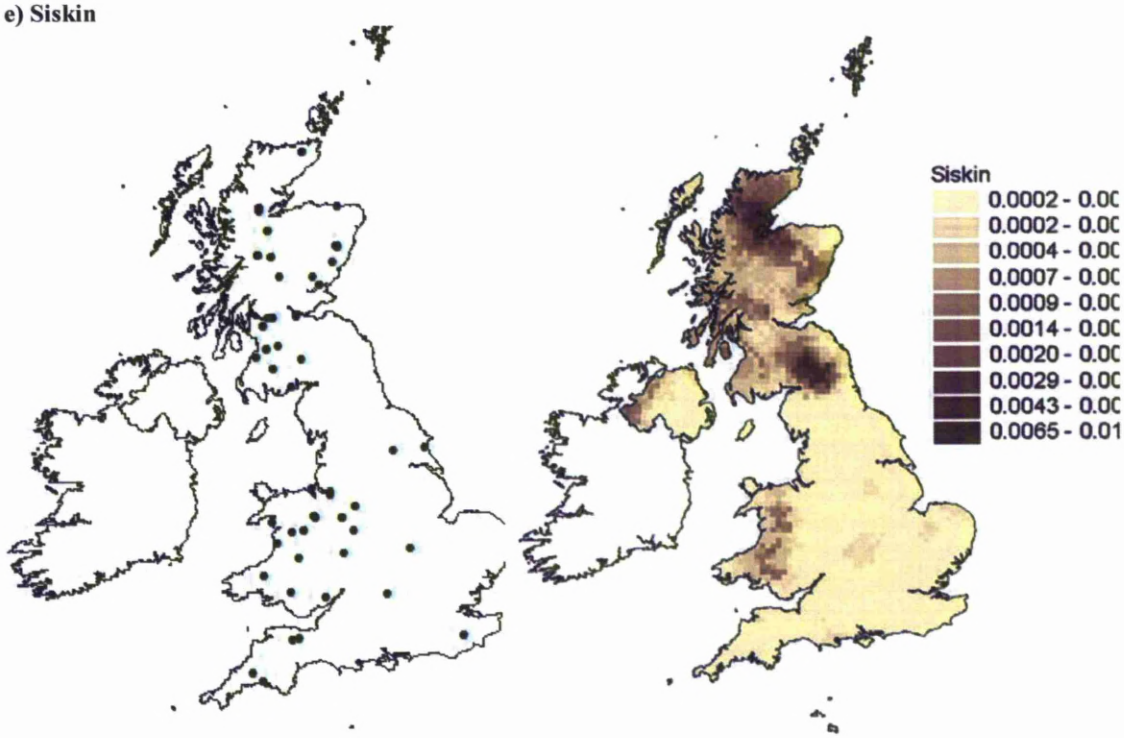
c) House Sparrow (BBS data not available)



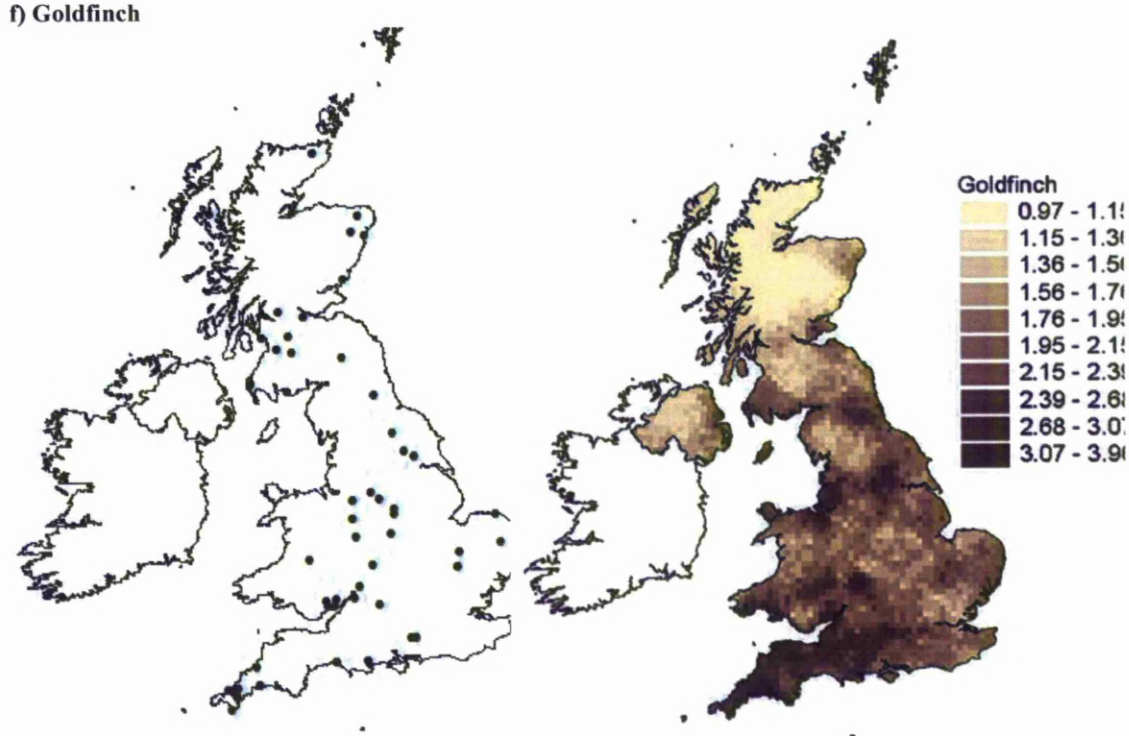
d) Blackbird



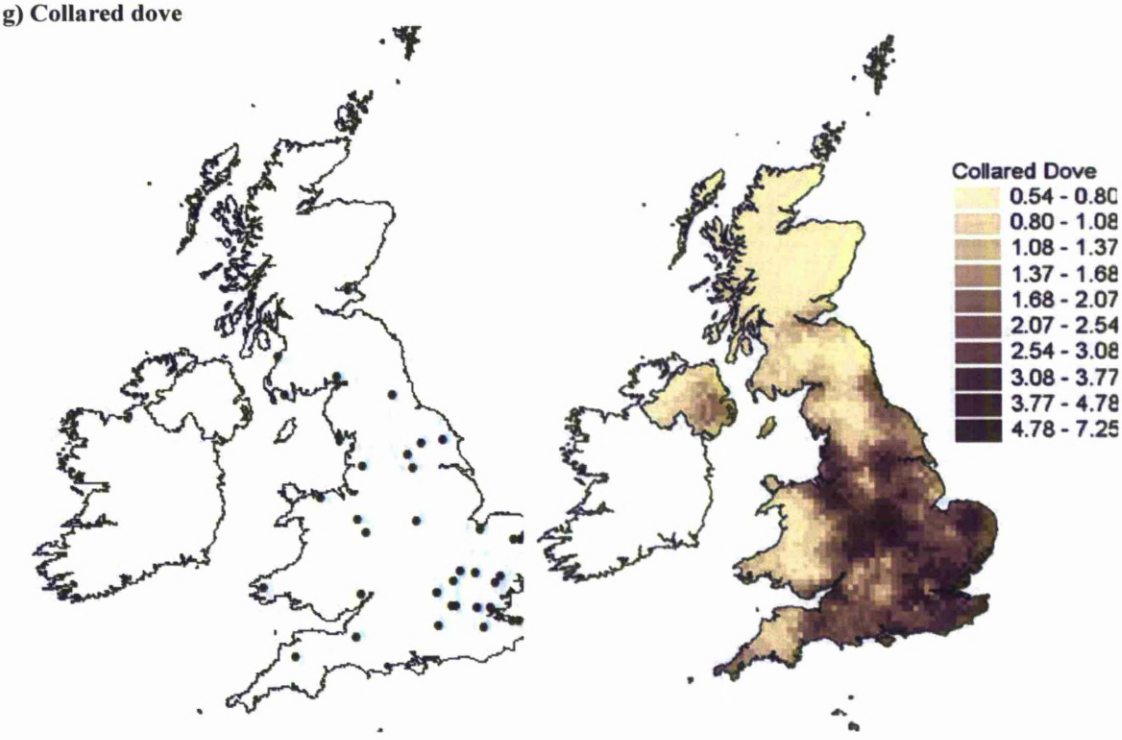
e) Siskin



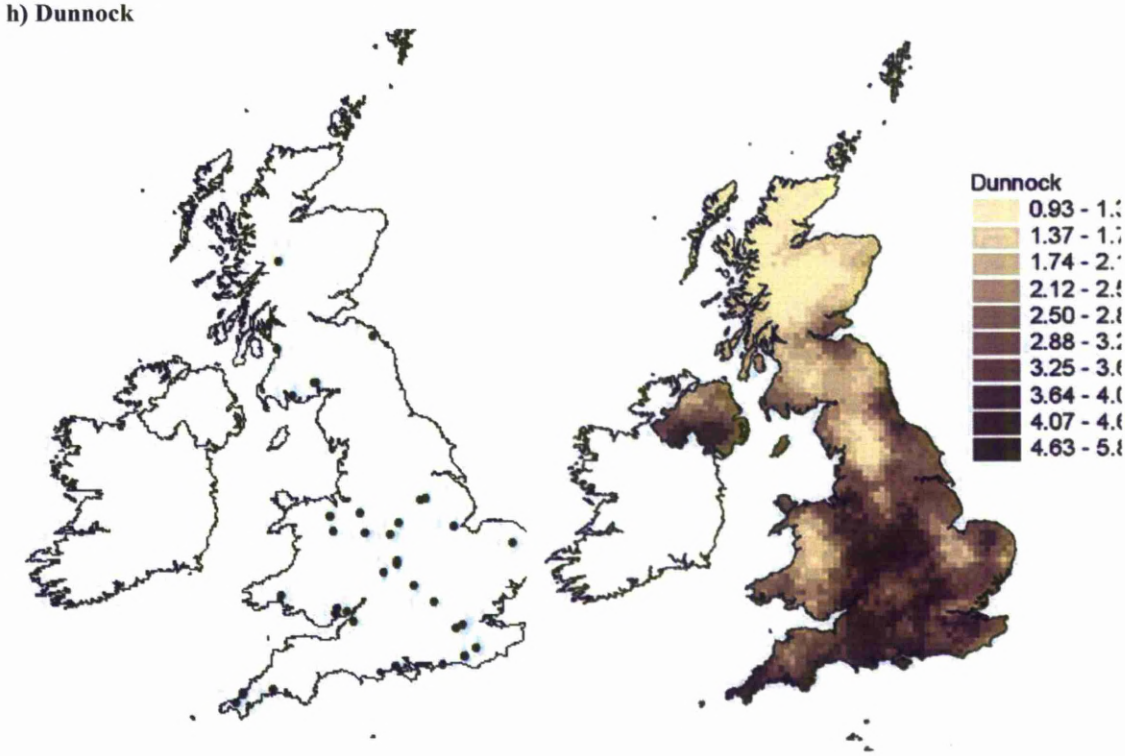
f) Goldfinch



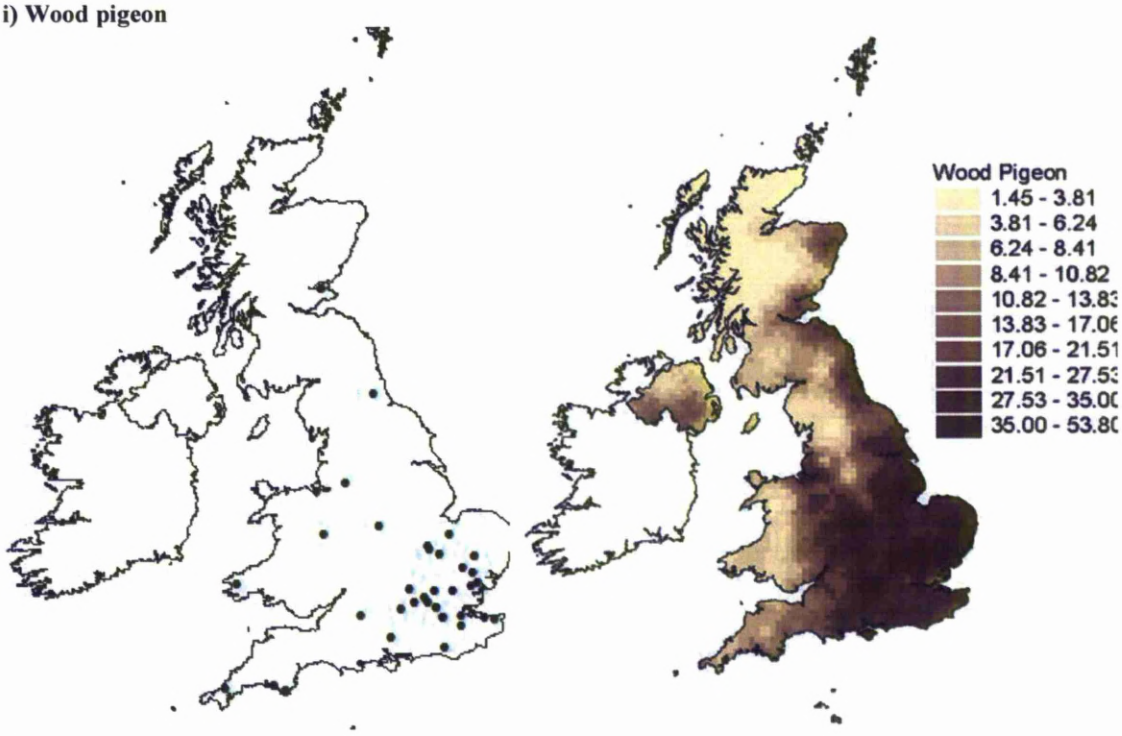
g) Collared dove



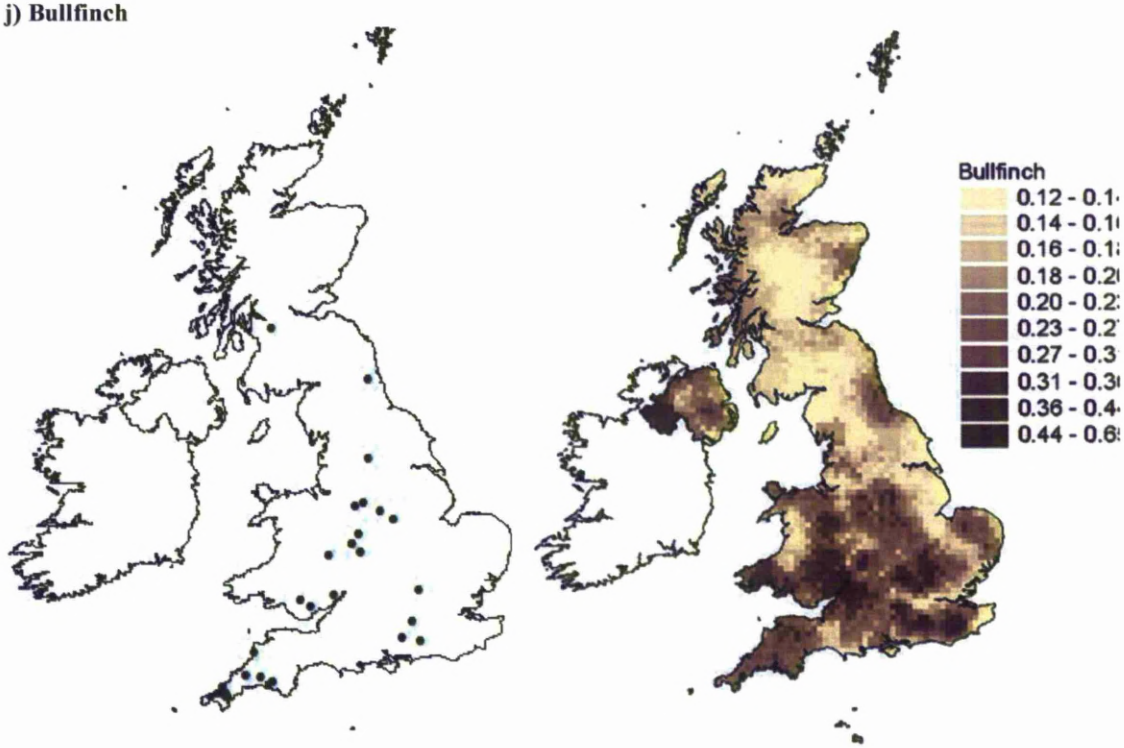
h) Dunnock



i) Wood pigeon



j) Bullfinch



Necrotic ingluvitis in wild finches

SIR, – Infection with *Salmonella enterica* serotype Typhimurium is a common cause of thickening, ulceration and necrosis of the oesophagus and crop of wild finches of the family Fringillidae (Pennycott and others 1998), with most cases occurring between January and April (Pennycott and others 2002). Profuse growths of *S* Typhimurium can be cultured from the viscera of affected birds.

Over the past year we have encountered several instances of deaths in chaffinches (*Fringilla coelebs*) and greenfinches (*Carduelis chloris*) in which a necrotic ingluvitis was apparent at postmortem examination, similar to that seen in finches with salmonellosis but from which no significant bacteria or yeasts could be cultured. Affected birds included a greenfinch in October 2004, a chaffinch in April 2005, two greenfinches in June 2005, two chaffinches and a greenfinch in July 2005, and four greenfinches and a chaffinch in August 2005. The birds were submitted from eight sites in Scotland and England.

Gross lesions on the mucosa of the oesophagus and crop varied from small, focal, yellow nodules, 2 to 4 mm in diameter, to a more diffuse yellow-orange thickening of the mucosa, to more severe masses of yellow, orange or white debris overlying areas of deep ulceration and

FIG 1: Lesions on the mucosa of the oesophagus and crop of a finch showing masses of orange debris overlying areas of deep ulceration

causing partial or complete obstruction of the upper digestive tract (Fig 1). Changes were often visible from the serosal surface of affected organs, and sometimes even through the skin of the bird. A small caseous nodule was noted in the oropharynx of one bird.

Histopathological examination of the oesophagus and crop of three chaffinches from two locations showed a severe ulcerative oesophagitis and ingluvitis, with structures suspected to be trichomonads close to the deep borders of the lesions. In addition, wet preparations from the borders of the necrotic areas in the crop of a greenfinch demonstrated large numbers of motile protozoa, possibly trichomonads.

Trichomoniosis of the upper digestive tract is a common problem in pigeons and doves of the order Columbiformes (Chitty 2003), in birds of prey of the order Falconiformes (Samour and others 1995), and in budgerigars (*Melopsittacus undulatus*) (Baker 1986). Trichomonads have also been demonstrated in the oesophagus and crop of domestic fowl (*Gallus gallus domesticus*) and pheasants (*Phasianus colchicus*) (Willoughby and others 1995, Pennycott 1998). However, to the best of our knowledge, this is the first time that trichomonads or similar organisms have been associated with necrotic ingluvitis in wild birds of the order Passeriformes, family Fringillidae, in the UK.

It is tempting to speculate that the finches may have acquired the organisms from other wild birds such as collared doves (*Streptopelia decaocto*), possibly at feeding stations or water baths provided by the general public, but evidence to support such a hypothesis has yet to be found. We have recently begun a national investigation (the Garden Bird Health initiative) into garden bird health and feeding practices (Cunningham and others 2005), and would be very interested to hear if other colleagues have encountered this condition in garden finches.

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Julian Chantrey, *Leahurst Veterinary Teaching Hospital, Liverpool University, Neston, Wirral CH64 7TE*

References

BAKER, J. R. (1986) Trichomoniasis, a major cause of vomiting in budgerigars. *Veterinary Record* **118,** 447-449

CHITTY, J. R. (2003) Pigeons and doves. In BSAVA Manual of Wildlife Casualties. Eds E. Mollineaux, R. Best, J. E. Cooper. Quedgley, BSAVA. pp 255-259

CUNNINGHAM, A. A., LAWSON, B., BENNETT, M., CHANTREY, J., KIRKWOOD, J. K., PENNYCOTT, T. W. & SIMPSON, V. (2005) Garden bird health. *Veterinary Record* **156,** 656

PENNYCOTT, T. W. (1998) Carriage of trichomonads, *Hexamita* species and *Blastocystis* species by adult pheasants. *Veterinary Record* **143,** 142-143

PENNYCOTT, T. W., CINDEREY, R. N., PARK, A., MATHER, H. A. & FOSTER, G. (2002) *Salmonella enterica* subspecies *enterica* serotype Typhimurium and *Escherichia coli* O86 in wild birds at two garden sites in south-west Scotland. *Veterinary Record* **151,** 563-567

PENNYCOTT, T. W., ROSS, H. M., MCLAREN, I. M., PARK, A., HOPKINS, G. F. & FOSTER, G. (1998) Causes of death of wild birds of the family Fringillidae in Britain. *Veterinary Record* **143,** 155-158

SAMOUR, J. H., BAILEY, T. A. & COOPER, J. E. (1995) Trichomoniasis in birds of prey (Order Falconiformes) in Britain. *Veterinary Record* **136,** 358-362

WILLOUGHBY, D. H., BICKFORD, A. A., CHARLTON, B. R. & COOPER, G. L. (1995) Esophageal trichomoniasis in chickens. *Avian Diseases* **39,** 919-924

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Exposure of garden birds to aflatoxins in Britain

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Abstract

Aflatoxins are potent biological toxins that have been shown to exert a range of acute and chronic pathological effects. Multiple mortality events of waterfowl caused by acute aflatoxicosis have been documented in the USA. However, international concern has recently been expressed regarding the potential effects of chronic exposure of wildlife species to low levels of dietary aflatoxin. This study documents for the first time the presence of hepatic aflatoxin residues in British wild birds: two passerine species, the house sparrow (*Passer domesticus*) and greenfinch (*Carduelis chloris*). Further research is required to investigate the source of the dietary aflatoxins and their pathological significance, if any, for wild birds in Britain.

Keywords: Aflatoxin; *Passer domesticus*; *Carduelis chloris*; Wild bird

1. Introduction

Aflatoxins are a group of compounds whose metabolites have been demonstrated to exert carcinogenic, immunosuppressive, hepatotoxic and other pathological effects. The ability of aflatoxins to impair protein synthesis and interact with metabolic pathways has been attributed to many of the above properties (Pier and Heddleston, 1970; Pier, 1992; Quist et al., 2000). The fungal species, *Aspergillus flavus, A. parasiticus* and *A. nomius* that occur commonly in the air, soil and other natural substrates, are responsible for the production of these toxins (Pitt and Hocking, 1997). Aflatoxins are considered natural contaminants of a variety of agricultural products, with corn, peanuts, cottonseed, and other grain crops being most frequently contaminated (Gourama and Bullerman, 1995; Creekmore, 1999).

Aflatoxins were first identified when they were found to be a cause of acute toxicity in commercial turkeys in the 1960s, the condition being known as "Turkey-X disease" (Blount, 1961). Aflatoxin toxicity has been documented since in a variety of taxa (mammals, fish and birds). Although birds appear to be the most susceptible (Creekmore, 1999), there is significant variation between avian species in their susceptibility to aflatoxins. Also, susceptibility to the effects of aflatoxins varies with age, sex and nutritional status, with young birds likely to be the most significantly affected (Creekmore, 1999; Maia and Pereira Bastos de Siqueira, 2002). The acute effects of aflatoxin toxicity are characterised by hepatic injury, coagulopathy, haemorrhage, icterus and death (Pier, 1992). Chronic, low level exposure to aflatoxins has been shown to be associated with a range of more insidious effects, such as reduced weight gain, suppression of the im-

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mune system, interference with reproductive function and neoplasia (Pier, 1992; Sharma, 1993; Ortatatli et al., 2002; Verma et al., 2004).

Much research has been performed on the effect of aflatoxin exposure on domestic farmed bird species (Muller et al., 1970). Given the susceptibility of farmed turkeys to aflatoxins, concern was raised over similar potential effects on wild turkeys (*Meleagris gallopova silvestris*) in the USA. Experimental aflatoxin exposure in this species led to reduced weight gain and feed consumption, impaired cell-mediated immunity and mild liver damage (Quist et al., 2000). Feeding trials were performed on wild game bird species to examine the degree of interspecific variation in their response to aflatoxin exposure. Dietary aflatoxin concentrations of 1250, 2500 and 5000 μg/kg (ppb) were fed for three weeks, since these represent the range of concentrations in poultry that have been found to result in no adverse effects, mild effects and severe effects, respectively. In order of relative susceptibility to the effects of aflatoxin, ring neck pheasants (*Phasianus colchicus*) were most affected, followed by the domestic chicken and bobwhite quail (*Colinus virginianus*); Chukar partridge (*Alectoris chukar*) and the Japanese quail (*Coturnix coturnix japonica*) were relatively resistant. Significant mortality was experienced by ring neck pheasants and bobwhite quail at dietary aflatoxin levels exceeding 1250 μg/kg (Ruff et al., 1990, 1992; Huff et al., 1992).

Historically, the majority of aflatoxin research has been performed because of the economic implications to the poultry industry of chronic exposure (Oliveira et al., 2002) or for evaluation of potential public health risks through consumption of contaminated meat, milk or eggs (Maia and Pereira Bastos de Siqueira, 2002). However, in recent years, increasing focus has been placed on the study of aflatoxicosis in free-ranging wildlife species. Mass mortalities of several waterfowl species due to acute aflatoxicosis have been reported in Texas and Louisiana, USA. Deaths occurred during the autumn/winter season and were attributed to the birds feeding on waste crops (e.g., peanuts, corn) in agricultural fields (Robinson et al., 1982; Cornish and Nettles, 1999; Creekmore, 1999). Robinson et al. (1982) reported two mortality incidents affecting primarily snow geese (*Anser caerulescens*) and mallards (*Anas platyrhynchos*), respectively. Analysis of a pooled sample of oesophageal and proventricular contents from affected birds in the first incident yielded 500 μg/kg AFB_1 (dry weight) whilst the same sample types from individual birds from the second incident yielded levels from 10 to 250 μg/kg AFB_1 (dry weight). Robinson et al. (1982) found 110 μg/kg AFB_1 in a sample of peanuts taken randomly from the field of the second incident. Cornish and Nettles (1999) analysed samples of corn from fields where a variety of goose species were affected by acute aflatoxicosis and found levels as high as 8200 μg/kg aflatoxin. Recently, concern has been expressed for the significance of chronic low level exposure of aflatoxins to wild bird species either through supplementary, agricultural or wild seed sources (Schweitzer et al., 2001).

Although no evidence, either anecdotal or scientific, is available on aflatoxin exposure of birds in Britain, such exposure has been repeatedly raised as a possible cause of morbidity or mortality of garden birds provisioned with commercially available food by householders. Consequently, high standards of food quality have been set for garden bird feed retailers belonging to voluntary associations, such as the Bird Care Standards Association, with members opting to follow a code of standards whereby only peanuts that have been screened and found to contain nil detectable levels of aflatoxin (BSA, 2005) are sold. Most food purchased for garden bird provisioning in Britain is, however, bought from retailers (such as independent pet shops) outside these associations, and aflatoxin B_1 levels in these products are set at the legal maximum permissible level (MPL) of 20 μg/kg, as is the case for all feeding stuffs in Britain (Anon, 2004a,b,c,d).

To investigate if aflatoxin exposure is a risk for garden birds in Britain, we conducted aflatoxin analyses on liver samples collected from a subset of garden birds submitted to the Institute of Zoology, London, for postmortem examination between 1999 and 2003. The liver was selected for aflatoxin testing because this organ retains the highest levels of aflatoxins following ingestion (Madden and Stahr, 1995) and is the major target organ in terms of the toxins' pathological effects (Oliveira et al., 2002).

2. Materials and methods

Opportunistic reports of garden bird mortality were solicited from members of the public through an organisational network including the British Trust for Ornithology (BTO), the Royal Society for the Protection of Birds (RSPB), the Royal Society for the Prevention of Cruelty to Animals (RSPCA), the Universities Federation of Animal Welfare (UFAW) and the Zoological Society of London (ZSL). Where available, carcasses were submitted to the Institute of Zoology where they

were examined following a standardised postmortem examination protocol. Details of the date found, geographical origin and circumstances were recorded. Each bird was assigned a unique postmortem reference code. During each examination, the species, age, sex, total body weight and body condition were recorded. Systematic internal and external examination of body systems was performed and gross lesions described. Where indicated, and where the state of carcass decomposition permitted, samples were taken for parasitological and toxicological investigation. No cases were considered to be fresh enough for meaningful histopathological examination.

Liver, small intestine and any lesions observed were routinely sampled aseptically and examined for the presence of pathogenic bacteria using a standard protocol. Briefly, tissue samples were plated directly onto (1) Colombia blood agar (QCM laboratories, Unit 205–206, Greenheath Business Centre, Three Colts Lane, London, E2 6JL, UK) supplemented with 5% horse blood, and incubated under aerobic, anaerobic and carbon dioxide conditions and observed after 1, 2 and 5 days (2) Xylose–Lysine Deoxycholate (XLD) medium agar (QCM laboratories) and incubated under aerobic conditions and observed after 2 days. (3) immersed into selenite *Salmonella*-selective enrichment broth (QCM laboratories) under aerobic conditions for 48 h followed by subculture onto XLD agar aerobically, 37 C for a further 48 h. (4) Chocolate blood agar (QCM Laboratories) in carbon dioxide at 37 C and observed after 1, 2 and 5 days. GENbox Anaerobic and CO_2 gas packs in AnaeroPack rectangular jars (bioMerieux, Marcy 1'Etoile, France) were used. Bacterial isolates were identified using colony and organism morphology, Gram's staining properties and biochemical properties using the API biochemical test strip method (Biomerieux BioMerieux, Marcy l'Etoile, France). Slide agglutination tests were performed for the identification of suspected *Salmonella* sp. isolates using poly-O antisera (Pro-lab diagnostics). Isolates were then placed onto microbank beads (Pro-lab diagnostics) and stored at both −25 and −70 °C. Batches of isolates were later submitted to the *Salmonella* Reference Unit, Laboratory of Enteric Pathogens, Health Protection Agency, for complete biotyping according to standardised international protocols (Anderson et al., 1977).

An archive of liver samples from garden birds was kept in frozen storage at −20 °C. Liver samples from 13 greenfinches (*Carduelis chloris*) (13 locations) and from 22 house sparrows (*Passer domesticus*) (16 locations) found dead between 1999 and 2003 (Table 1) were analysed at the Central Science Laboratory for the major aflatoxin residues (AFB_1, AFB_2, AFG_1, AFG_2) using immunoaffinity column (IAC) clean-up and high pressure liquid chromatography. The method employed was based on published methods for aflatoxins (Sharman and Gilbert, 1991). Briefly, samples were weighed and chloroform and 0.1 M phosphoric acid solution were added before the samples were blended at high speed using an Ultra turrax blender. The samples were centrifuged and the chloroform extract (or an aliquot of it) was evaporated to dryness using a rotary evaporator. The residue was redissolved in methanol and phosphate buffered saline (PBS). This was partitioned with hexane in a separating funnel. An aliquot of the methanol/PBS extract was applied to an Easi-Extract aflatoxin immunoaffinity column (R-Biopharm Rhone Ltd., Glasgow), preconditioned with PBS. The columns were washed with distilled water (10 ml) and then eluted using high performance liquid chromatography (HPLC) grade acetonitrile (1.5 ml). An aliquot of the cleaned up acetonitrile extract was diluted with water prior to analysis by reversed phase HPLC. The IAC clean-up and HPLC injection were carried out automatically by a Gilson ASPEC system fitted with an automated Rheodyne switching valve and injection loop. Injection was by partial loop fill, using an injection volume of 400 µl. The HPLC system also comprised a Gilson HPLC pump, and a JASCO FP 1520 fluorescence detector set at excitation wavelength 364 nm and emission wavelength 440 nm. Chromatography was performed using a Spherisorb ODS1-Excel HPLC column, (25 cm × 4.6 mm i.d., 5 µm particle size). The mobile phase consisted of water:acetonitrile:methanol, (54:30:16, v/v/v) at a flow rate of 1.0 ml/min. Pyridinium bromide perbromide (PBPB, 50 mg/l, pumped at 0.3 ml/min) was used for post column derivatisation of aflatoxins B_1 and G_1.

Quantification was by external calibration. Fresh calibration standards were prepared for each batch of samples. These were injected at the beginning and end of each run and were also interspersed between samples. All standards in a run were used to prepare four point calibration curves. Peaks were identified by matching retention times to the nearest calibration standard. The specificity of the clean-up, derivatisation method and specific excitation and emission wavelengths used were considered sufficient to ensure accurate peak identification. Duplicate spiked samples were analysed in each batch, recovery values were in the range 70–93% (acceptable range 70–110%). All results met quality assurance parameters e.g., peak asymmetry, retention time drift, resolution etc. established for UKAS accredited aflatoxin analyses.

Table 1
Results of aflatoxin screening of liver samples from garden birds

No	Species	Season and year of death	Cause of death	Aflatoxin residues measured† μg/kg (ppb)				
				AFB1	AFB2	AFG1	AFG2	Total AF
1	Greenfinch	Winter 1999/2000	Salmonellosis	0.1	ND	ND	ND	0.1
2	House sparrow	Summer 2000	Other trauma	ND	ND	ND	ND	ND
3	House sparrow	Autumn 2000	Salmonellosis	0.8	0.1	ND	ND	0.9
4	House sparrow	Autumn 2000	Salmonellosis	0.4	ND	ND	ND	0.4
5	Greenfinch	Winter 2000/2001	Salmonellosis	ND	ND	ND	ND	ND
6	Greenfinch	Winter 2000/2001	Salmonellosis	ND	ND	ND	ND	ND
7	Greenfinch	Spring 2001	Other trauma	ND	ND	ND	ND	ND
8	Greenfinch	Winter 2001/2002	Salmonellosis	0.3	ND	ND	ND	0.3
9	Greenfinch	Winter 2001/2002	Salmonellosis	ND	ND	ND	ND	ND
10	Greenfinch	Winter 2001/2002	Not established	ND	ND	5.8	2.2	8.0
11	Greenfinch	Winter 2001/2002	Salmonellosis	0.1	ND	ND	ND	0.1
12	Greenfinch	Winter 2001/2002	Salmonellosis	0.2	ND	ND	ND	0.2
13	Greenfinch	Winter 2001/2002	Salmonellosis	0.1	ND	ND	ND	0.1
14	Greenfinch	Winter 2001/2002	Salmonellosis	0.7	0.1	ND	ND	0.8
15	House sparrow	Winter 2001/2002	Salmonellosis	ND	ND	ND	ND	ND
16	House sparrow	Winter 2001/2002	Salmonellosis	ND	ND	ND	ND	ND
17	House sparrow	Winter 2001/2002	Salmonellosis	ND	ND	ND	ND	ND
18	House sparrow	Winter 2001/2002	Salmonellosis	ND	ND	ND	ND	ND
19	House sparrow	Winter 2001/2002	Salmonellosis	ND	ND	ND	ND	ND
20	House sparrow	Winter 2001/2002	Salmonellosis	0.4	ND	ND	ND	0.4
21	House sparrow	Winter 2001/2002	Salmonellosis	0.4	ND	ND	ND	0.4
22	Greenfinch	Spring 2002	Predation	ND	ND	ND	ND	ND
23	House sparrow	Spring 2002	Predation	ND	ND	ND	ND	ND
24	House sparrow	Spring 2002	Predation	ND	ND	ND	ND	ND
25	House sparrow	Spring 2002	Predation	ND	ND	ND	ND	ND
26	House sparrow	Spring 2002	Predation	ND	ND	ND	ND	ND
27	Greenfinch	Summer 2002	Predation	ND	ND	ND	ND	ND
28	House sparrow	Summer 2002	Predation	ND	ND	ND	ND	ND
29	House sparrow	Summer 2002	Predation	ND	ND	ND	ND	ND
30	House sparrow	Summer 2002	Predation	ND	ND	ND	ND	ND
31	House sparrow	Summer 2002	Predation	ND	ND	ND	ND	ND
32	House sparrow	Summer 2002	Predation	ND	ND	ND	ND	ND
33	House sparrow	Autumn 2002	Predation	ND	ND	ND	ND	ND
34	House sparrow	Summer 2003	Predation	ND	ND	ND	ND	ND
35	House sparrow	Summer 2003	Predation	ND	ND	ND	ND	ND

†ND = not detectable.

Analyses were performed in two batches with variable residue detection limits according to sample volume and equipment calibration. Greenfinches and house sparrows were chosen for analysis as these two species are among the most common granivorous garden birds in Britain, are commonly submitted for postmortem examination and represent species that have increasing (greenfinch) and declining (house sparrow) populations (Baillie et al., 2004).

The aflatoxin analyses results were explored by cause of death and by season of death: winter (Dec–Feb), spring (Mar–May), summer (Jun–Aug) or autumn (Sept–Nov) (Table 1). Geographical location data was explored using ArcView 3.0 software (ESRI GIS and Mapping Software, 380 New York Street, Redlands, CA 92373-8100, USA).

3. Results

The results of the postmortem examinations are summarised in Table 1. Cause of death was categorised as, in decreasing order of occurrence: "salmonellosis", "predation", "other trauma" and "not established". All birds in this study that died of infectious disease did so of salmonellosis (nine greenfinches, nine house sparrows). No other infectious cause of death was found.

The causes of death reflect those most commonly found for greenfinches and house sparrows in a review of eleven years of garden bird postmortem examinations at the Institute of Zoology (B.L. and A.A.C., unpublished results). Salmonellosis was diagnosed on the basis of gross postmortem findings consistent with

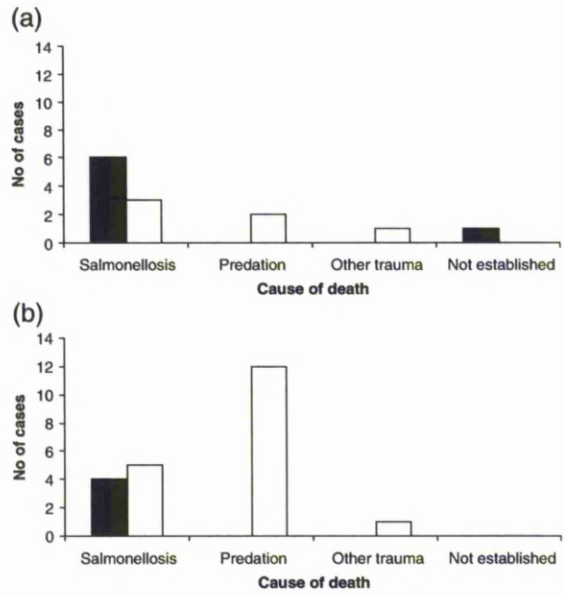

Fig. 1. Number of (a) greenfinches ($n=13$) and (b) house sparrows ($n=22$) screened for aflatoxin residues by cause of death. Black columns represent birds positive for residues. White columns represent birds negative for residues.

salmonellosis coupled with the isolation of *Salmonella* Typhimurium from lesions (Pennycott et al., 1998). None of the birds that died from predation or other trauma (three greenfinches, 13 house sparrows) had any evidence of infectious or other concurrent disease processes on pathological examination, including an absence of significant findings obtained on microbiological examination. These cases, therefore, have been considered together as a "non-infectious" cause of death group. The cause of death of one greenfinch could not be determined. This bird was not assigned to either the "infectious disease" or the "non-infectious" group.

Aflatoxin residues above detectable thresholds were identified in four of 22 house sparrows and in seven of 13 greenfinches examined (Table 1). Aflatoxin B_1 was the principal component in all but one of these eleven positive cases, with values ranging between 0.1–0.8 μg/kg. Aflatoxin B_2 was also detected in the two cases with the highest levels of AFB_1. No gross evidence of hepatic disease consistent with either acute or chronic aflatoxicosis was observed in the birds examined.

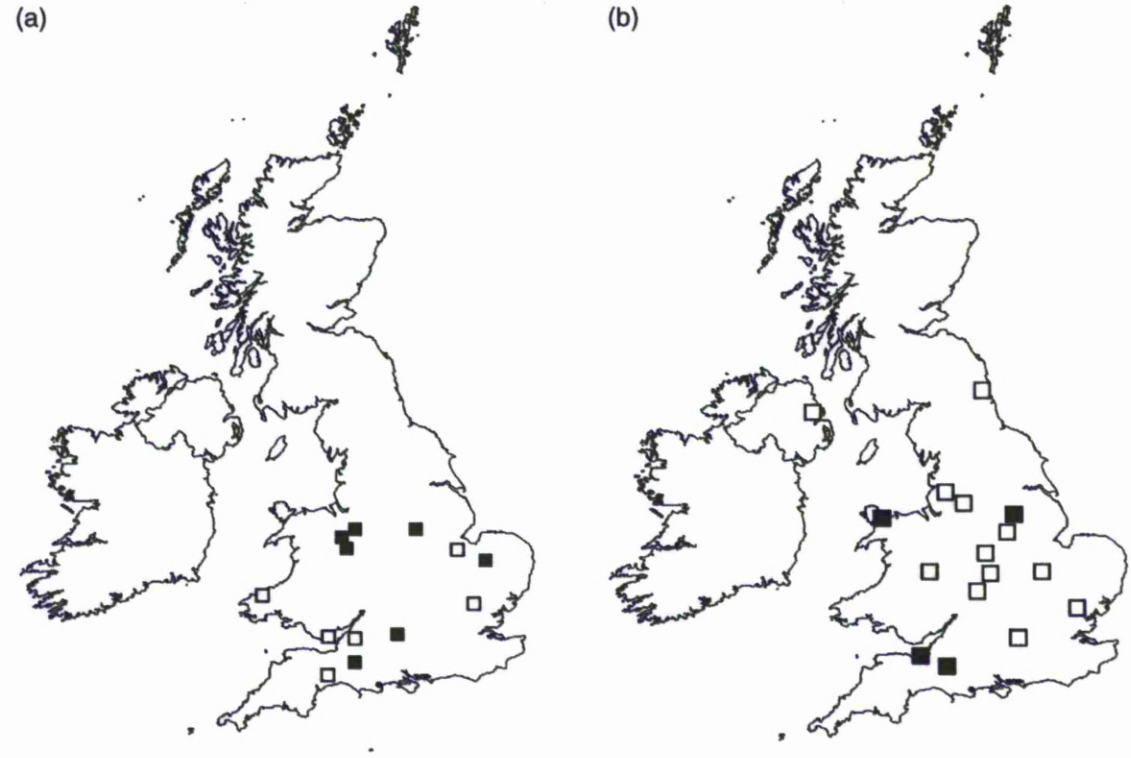

Fig. 2. Geographical distribution of (a) greenfinches ($n=13$ locations) and (b) house sparrows ($n=16$ locations) screened for aflatoxin residues. Each bold square represents the location of a bird that tested positive for residues. Each empty square represents the location of a bird (or birds) that tested negative for residues.

AFG_1 and AFG_2 residues were found in one greenfinch only, with a total hepatic aflatoxin level of 8.0 μg/kg. No detectable levels of AFB_1 or AFB_2 were found in this case. Unfortunately, the cause of death could not be established for this bird due to the extent of carcass decomposition; gross and microbiological examinations were negative for evidence of infectious disease, such as salmonellosis.

For both species examined, AFB_1 and AFB_2 residues were detected only in birds that had died as a result of infectious disease (salmonellosis). All tissues examined from birds in the "non-infectious disease" group (i.e., birds that had died as a result of predation or other trauma) were negative for aflatoxin residues (Fig. 1).

Only a small number of birds were examined in this study, but although there was no evidence of geographical (Fig. 2), or temporal clustering of cases with detectable aflatoxin residues there was a strong seasonal influence. Detectable levels of aflatoxin were found only in birds that died between November and February (Fig. 3). Detectable residues were found in birds that died in 1999 (1 of 1 case), 2000 (2 of 5 cases) and 2001 (8 of 15 cases), whilst no residues were detected in birds examined in 2002 (0 of 12 cases) or 2003 (0 of 2 cases).

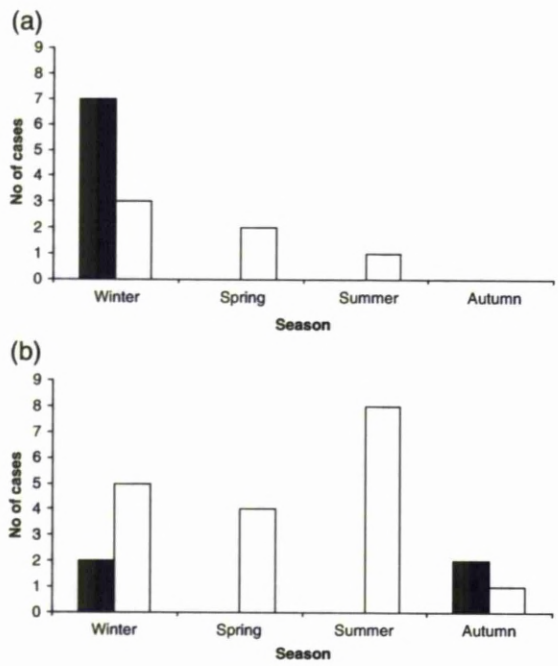

Fig. 3. Number of a) greenfinches ($n=13$) and b) house sparrows ($n=22$) screened for aflatoxin residues by season. Bold columns represent birds positive for residues. White columns represent birds negative for residues. Winter (Dec–Feb); Spring (Mar–May); Summer (June–Aug); Autumn (Sept–Nov).

4. Discussion

Hepatic aflatoxin residues were identified in four of 22 house sparrows and in seven of 13 greenfinches examined. To the authors' knowledge, this is the first report of wild birds being exposed to aflatoxins in Britain.

Aflatoxin B_1 is the most commonly occurring and the most toxic of the group of aflatoxins (Creekmore, 1999) and this was the toxin most frequently identified in the current study. The order of toxicity of the remaining aflatoxins is AFG_1, greater than AFB_2, and AFG_2 in sequence (Gourama and Bullerman, 1995). The presence of relatively high levels of AFG_1 and AFG_2 in a single greenfinch, in the absence of other residues, is of note. Aflatoxin B_2 was detected in the two birds with the highest levels of AFB_1.

The absence of any gross evidence of toxicity and the inability to conduct meaningful histopathological examinations on the birds tested for aflatoxin exposure makes interpretation of the results difficult. There is little reported in the literature on hepatic aflatoxin levels in birds and the majority of these reports are based on experimental dosing of avian species (domestic and wild) with known dietary concentrations of aflatoxins followed by monitoring of the physiological or pathological effects observed. Little information is available regarding the relationships between dietary aflatoxin levels and resultant tissue aflatoxin residues for bird species, particularly in combination with an assessment of the aflatoxins' pathological significance.

In some studies, however, correlations have been made between dietary aflatoxin concentrations and the subsequent concentrations of tissue residues, although often the latter are at very low levels, in the order of < several micrograms per kilogram (Gregory et al., 1983). Bintvihok et al. (2002) fed a variety of domestic poultry species with diets containing 3000 μg/kg AFB_1 for a 7-day period and showed that levels of AFB_1 and its metabolites were greater in liver than in muscle for all bird species tested. The ratio of AFB_1 toxin in the feed to the residual level in the liver varied between 383:1 for quail and ≥5769:1 for ducks and chickens. Madden and Stahr (1995) fed 700 μg/kg AFB_1 to chicks for 28 days and demonstrated that the highest levels of AFB_1 were found in the liver (1.29 μg/kg), followed by the crop (0.074 μg/kg) and muscle tissue (0.014 μg/kg) with an aflatoxin conversion factor from feed to liver of around 543:1. In another experiment to evaluate rates of aflatoxin residue clearance, Gregory et al. (1983) fed day-old turkey poults on a diet containing 500 μg/kg of AFB_1 for a period of 18 days, followed by

a variable withdrawal period on a control diet. Results showed that tissue levels of aflatoxin residue were greater in liver than in muscle tissue, although all tissue residues were at a low level (range 0.01–1.19 μg/kg). Postmortem examination of the poults on this diet revealed slight liver hypertrophy, mild petechial haemorrhages and occasional tan discolouration of the liver.

These aflatoxin feeding experiments indicate that the metabolism of dietary aflatoxin extrapolates to a much smaller corresponding level in liver tissues with a conversion ratio that is highly variable according to species and other factors. Consequently, it is likely that the levels of hepatic aflatoxin residues found in British wild birds in the current study result from a much higher dietary level of exposure.

Dietary aflatoxins are rapidly metabolised and excreted from the body, both in free and conjugated forms. For example, a study in turkey poults determined a half-life of 1.4 days for total aflatoxin residues in the liver (Gregory et al., 1983). Whilst the half-life for hepatic aflatoxins in the house sparrow and greenfinch are unknown, the rapid elimination in other avian species suggests that the birds in this study were exposed to contaminated dietary sources in the recent period prior to death. The exposure of garden birds in Britain to aflatoxins, therefore, is likely to be more frequent and widespread than our results indicate.

Chronic aflatoxin exposure has been shown to produce histopathological abnormalities such as mild biliary hyperplasia, periportal fibrosis and hepatocellular lipidosis (Pier, 1992; Ortatatli et al., 2005). Appraisal of liver histopathology in tandem with aflatoxin screening would be a logical next stage in determining the significance of aflatoxin exposure in British wild birds.

It has been hypothesised that immunosuppression, predisposing to infectious disease, might be one of the most important effects of aflatoxins on wild turkeys (Quist et al., 2000). This might also be true for other free-ranging species exposed to sub-lethal doses of aflatoxins. In the current study, AFB_1 residues were detected only in garden birds that had died as a consequence of infectious disease (salmonellosis) and it is tempting to speculate that this could be due to aflatoxin-mediated immunosuppression. Devegowda and Murthy (2005) specifically cite increased susceptibility to salmonellosis as a consequence of subacute aflatoxicosis in poultry. However, salmonellosis has a highly seasonal occurrence in garden birds in Britain, peaking during the winter months (Pennycott et al., 1998; B.L. and A.A.C. unpublished data) and all AFB_1-positive birds in the current study died between November and February. The toxicological examination of birds that die of non-infectious causes in the winter months and of birds that die of infectious disease during the rest of the year are required in order to separate the confounding variables of infectious disease and season. Also, further research is required to determine the source of aflatoxins for garden birds in Britain. A study by Scudamore et al. (1997), for example, found evidence of aflatoxin contamination in one of fifteen samples of wild bird food screened in the UK. It might be that exposure of garden birds to aflatoxins is greater during the winter, perhaps through their increased reliance on supplementary feeding during this period. Further research is required to investigate the source of the dietary aflatoxins and their pathological significance, if any, for wild birds in Britain.

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References

Anderson ES, Ward LR, de Saxe MJ, De Sa JDH. Bacteriophage-typing designations of *Salmonella typhimurium*. J Hyg 1977; 78:297–300.

Anon. The feeding stuffs, the feeding stuffs (sampling and analysis) and the feeding stuffs (enforcement) (amendment) (England) (No2) regulations 2004. Statutory Instrument No. 2688, HMSO, 2004a, p. 1–12.

Anon. The feeding stuffs, the feeding stuffs (sampling and analysis) and the feeding stuffs (enforcement) (amendment) regulations (Northern Ireland) 2004. Statutory Rule No. 443, HMSO, 2004b, p. 1–11.

Anon. The feeding stuffs, the feeding stuffs (sampling and analysis) and the feeding stuffs (enforcement) (amendment) (Wales) (No 2) regulations 2004. Welsh Statutory Instrument No. 3091, HMSO, 2004c, p. 1–12 W.265.

Anon. The feeding stuffs (Scotland) amendment (No 2) regulations 2004. Scottish Statutory Instrument 2004 No. 458, HMSO, 2004d, p. 1–10.

Baillie SR, Marchant JH, Crick HQP, Noble DG, Balmer DE, Beaven LP, et al. Breeding birds in the wider countryside: their conservation status 2004. BTO Research Report No. 385 2005. Thetford: BTO; 2005 http://www.bto.org/birdtrends.

Bintvihok A, Thiengnin S, Doi K, Kumagai S. Residues of aflatoxins in the liver, muscle and eggs of domestic fowls. J Vet Med Sci 2002;64(11):1037–9.

BirdCare Standards Association (BSA). Feeds Standards. http://www.birdcare.org.uk (Accessed May 2005).

Blount WP, 1961. Turkey "X" disease. Turkeys 9(2): 52, 55–58, 61–71, 77.

Cornish TE, Nettles Jr VF. Aflatoxicosis in Louisiana geese. SCWDS Briefs 1999;15(1):1–2.

Creekmore LH. Mycotoxins. In: Friend M, Franson JC, editors. Field Manual of Wildlife Diseases, U.S. Department of the Interior, U.S. Geological Survey, 1999, 267–270.

Devegowda G, Murthy TNK. Mycotoxins. Their effects in poultry and some practical solutions. In: Diaz D, editor. The mycotoxin blue book. Nottingham University Press, 2005, p. 30.

Gourama H, Bullerman LB. *Aspergillus flavus* and *Aspergillus parasiticus:* aflatoxigenic fungi of concern in foods and feeds: a review. J Food Protect 1995;58(12):1395–404.

Gregory JF, Goldstein SL, Edds GT. Metabolite distribution and rate of residue clearance in turkeys fed a diet containing aflatoxin B_1. Food Chem Toxicol 1983;21(4):463–7.

Huff WE, Ruff MD, Chute MB. Characterisation of the toxicity of the mycotoxins aflatoxin, ochratoxin, and T-2 toxin in game birds II Ringneck pheasant. Avian Dis 1992;36:30–3.

Madden UA, Stahr HM. Retention and distribution of aflatoxin in tissues of chicks fed aflatoxin-contaminated poultry rations amended with soil. Vet Hum Toxicol 1995;37(1):24–9.

Maia PP, Pereira Bastos de Siqueira ME. Occurrence of aflatoxins B_1, B_2, G_1 and G_2 in some Brazilian pet foods. Food Addit Contam 2002;19(12):1180–3.

Muller RD, Carlson CW, Semeniuk G, Harshfield GS. The response of chicks, ducklings, goslings, pheasants, and poults to graded levels of aflatoxins. Poultry Sci 1970;49(5):1346–50.

Oliveira CAF, Rosmaninho JF, Butkeraitis P, Correa B, Reis TA, Guerra JL, et al. Effect of low levels of dietary aflatoxin B1 on laying Japanese quail. Poultry Sci 2002;81:976–80.

Ortatatli M, Ciftci MK, Tuzcu M, Kaya A. The effects of aflatoxin on the reproductive system of roosters. Res Vet Sci 2002;72:29–36.

Ortatatli M, Oğuz H, Hati poğlu F, Karaman M. Evaluation of pathological changes in broilers during chronic aflatoxin (50 and 100 ppb) and clinoptilolite exposure. Res Vet Sci 2005; 78(1):61–8.

Pennycott TW, Ross HM, McLaren IM, Park A, Hopkins GF, Foster G. Causes of death of wild birds of the family Fringillidae in Britain. Vet Rec 1998;143(6):155–8.

Pier AC. Major biological consequences of aflatoxicosis in animal production. J Anim Sci 1992;70:3964–7.

Pier AC, Heddleston KL. The effect of aflatoxin on immunity in turkeys I Impairment of actively acquired resistance to bacterial challenge. Avian Dis 1970;14(4):797–809.

Pitt JI, Hocking AD. Fungi and food spoilage. Second edition. Blackie Academic & Professional, 1997, pp. 377–83, 396–7.

Quist CF, Bounous DI, Kilburn JV, Nettles VF, Wyatt RD. The effect of dietary aflatoxin on wild turkey poults. J Wildlife Dis 2000; 36(3):436–44.

Robinson RM, Ray AC, Reagor JC, Holland LA. Waterfowl mortality caused by aflatoxicosis in Texas. J Wildlife Dis 1982; 18(3):311–2.

Ruff MD, Huff WE, Wilkins GC. Characterisation of the toxicity of the mycotoxins aflatoxin, ochratoxin, and T-2 toxin in game birds I Chukar partridge. Avian Dis 1990;34:717–20.

Ruff MD, Huff WE, Wilkins GC. Characterisation of the toxicity of the mycotoxins aflatoxin, ochratoxin, and T-2 toxin in game birds III Bobwhite and Japanese quail. Avian Dis 1992;36:34–9.

Schweitzer SH, Quist CF, Grimes GL, Forster DL. Aflatoxin levels in corn available as wild turkey feed in Georgia. J Wildlife Dis 2001; 37(3):657–9.

Scudamore KA, Hetmanski MT, Nawaz S, Naylor J, Rainbird S. Determination of mycotoxins in pet foods sold for domestic pets and wild birds using linked-column immunoassay clean-up and HPLC. Food Addit Contam 1997;14(2):175–86.

Sharma RP. Immunotoxicity of mycotoxins. J Dairy Sci 1993; 76:892–7.

Sharman M, Gilbert J. Automated aflatoxin analysis of foods and animal feeds using immunoaffinity column clean-up and high-performance liquid chromatographic determination. J Chromatogr 1991;543(1):220–5.

Verma J, Johri TS, Swain BK, Ameena S. Effect of graded levels of aflatoxin, ochratoxin and their combinations on the performance and immune response of broilers. Brit Poultry Sci 2004;45(4): 512–8.

Epidemic finch mortality

SIR, – Over recent weeks, the Garden Bird Health Initiative (GBHi) has received an unusual increase in the number of calls from members of the public reporting finch morbidity and mortality in their gardens. The greenfinch (*Carduelis chloris*) and chaffinch (*Fringilla coelebs*) are the species most frequently involved, sometimes in large numbers, and the national surveillance scheme has collated reports from a wide geographical area.

Postmortem investigation has identified necrotic oesophagitis and ingluvitis associated with trichomonad-like parasites. This appears to be a novel and growing epidemic, following on from an apparent initial emergence in 2005 (Cousquer 2005, Holmes and Duff 2005, Pennycott and others 2005) and, if we are to determine the underlying factors leading to its emergence and impact on garden bird species, further research is required. We would be grateful if colleagues could inform us of garden bird mortality incidents by calling the GBHi on 020 7449 6685. A factsheet about this condition and information on best feeding practice for garden birds is available on the UFAW website (www.ufaw.org.uk).

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References

COUSQUER, G. (2005) Ingluvitis and oesophagitis in wild finches. *Veterinary Record* **157,** 455

HOLMES, P. & DUFF, P. (2005) Ingluvitis and oesophagitis in wild finches. *Veterinary Record* **157,** 455

PENNYCOTT, T. W., LAWSON, B., CUNNINGHAM, A. A., SIMPSON, V. & CHANTREY, J. (2005) Necrotic ingluvitis in wild finches. *Veterinary Record* **157,** 360

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Characterisation of *Salmonella enterica* serotype Typhimurium isolates from wild birds in northern England from 2005 – 2006

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Abstract

Background: Several studies have shown that a number of serovars of *Salmonella enterica* may be isolated from wild birds, and it has been suggested that wild birds may play a role in the epidemiology of human and livestock salmonellosis. However, little is known about the relationship between wild bird *S. enterica* strains and human- and livestock- associated strains in the United Kingdom. Given the zoonotic potential of salmonellosis, the main aim of this study was to investigate the molecular epidemiology of *S. enterica* infections in wild birds in the north of England and, in particular, to determine if wild bird isolates were similar to those associated with disease in livestock or humans.

Results: Thirty two *Salmonella enterica* isolates were collected from wild birds in northern England between February 2005 and October 2006, of which 29 were *S. enterica* serovar Typhimurium (*S.* Typhimurium); one *S.* Newport, one *S.* Senftenberg, and one isolate could not be classified by serotyping. Further analysis through phage typing and macro-restriction pulsed-field gel electrophoresis indicated that wild passerine deaths associated with salmonellosis were caused by closely-related *S.* Typhimurium isolates, some of which were clonal. These isolates were susceptible to all antimicrobials tested, capable of invading and persisting within avian macrophage-like HD11 cells *in vitro*, and contained a range of virulence factors associated with both systemic and enteric infections of birds and mammals. However, all the isolates lacked the *sopE* gene associated with some human and livestock disease outbreaks caused by *S.* Typhimurium.

Conclusion: The wild bird isolates of *S. enterica* characterised in this investigation may not represent a large zoonotic risk. Molecular characterisation of isolates suggested that *S.* Typhimurium infection in wild passerines is maintained within wild bird populations and the causative strains may be host-adapted.

Background

Many studies have shown that a range of *Salmonella enterica* serovars can be isolated from both dead [1-4] and live wild birds [2,5-8] and several studies have suggested that wild birds are important in the epidemiology of human and livestock salmonellosis [9-11]. A number of these studies have used phenotypic methods to characterise isolates, including serotyping, phage typing and antimicrobial sensitivity profiling coupled with epidemiological analysis. These are all useful approaches, but the techniques used to differentiate isolates mean that conclusions that can be drawn from such studies are sometimes limited.

Although there is evidence to suggest that certain strains of *S. enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) are associated with different groups of wild birds [3,4] it is not known if the *Salmonella* strains that cause mortality in UK wild birds, particularly garden birds, are the result of strains belonging to the same clones, a limited range of strains or many different strains, perhaps with differing host and/or geographic ranges. In addition, little is known about the relationship between wild bird *Salmonella* strains and human and livestock-associated strains in the UK, particularly with respect to the virulence genes they contain and their antimicrobial sensitivity profiles. As salmonellosis in wild birds may be zoonotic or transmissible to livestock, it would be of value to understand these relationships in greater detail to assess the risk of wild birds as reservoirs or vectors of *Salmonella* infections.

The main aim of this study was to investigate the molecular epidemiology of *S. enterica* isolates from wild birds in the north of England and, in particular, to determine whether or not the characteristics of 'wild bird' isolates were similar to those of isolates associated with disease in livestock, in particular poultry, or human cases. The relatedness of isolates was determined through a combination of phage typing and macro-restriction pulsed-field gel electrophoresis (PFGE). The presence of a range of virulence-associated genes involved in both avian systemic disease and enteritis in mammals was also determined by PCR (virulotyping). The ability of *Salmonella* strains to invade and survive in host macrophages, an *in vitro* correlate of the ability to cause systemic infection, was determined, and the susceptibility to antimicrobials was determined for each isolate.

Results

Bacterial isolates

Thirty two *Salmonella enterica* isolates were collected from wild birds in Northern England from February 2005 until October 2006. *Salmonella* Typhimurium was the most common serotype identified (n = 29), of which the most common definitive bacteriophage type (DT) was DT 56 (n = 23) followed by DT 40 (n = 3), DT 41 (n = 2) and phage type (PT) U277 (n = 1). The *S.* Typhimurium isolates were from 9 greenfinches (*Carduelis chloris*), 8 Eurasian siskins (*Carduelis spinus*), 6 house sparrows (*Passer domesticus*), 2 goldfinches (*Carduelis carduelis*), 2 common starlings (*Sturnus vulgaris*), 1 collared dove (*Streptopelia decaocto*) and 1 wood pigeon (*Columba palumbus*). *S.* Newport (n = 1) and *S.* Senftenberg (n = 1) were isolated from a black-headed gull (*Larus ridibundus*) and a herring gull (*Larus argentatus*) respectively. One further isolate from a herring gull could not be classified by serotyping. Nineteen infected birds were female, 10 were male and the sex of 3 birds was not determined. Twelve birds were juveniles (in their first year), 11 were adults, and nine could not be aged.

The majority of isolates were from dead birds, but three live birds; one house sparrow and two common starlings also yielded *Salmonella* from their faeces. None of these three birds had obvious clinical signs associated with salmonellosis. All isolates were collected between August and April, with a peak of isolations in January (35%) and February (23%): no *Salmonella* was isolated from dead or live birds between May and July (Table 1).

Post-mortem examination

Post-mortem examinations were performed on 26 of the 29 dead birds. Twenty three of these birds (88%) were considered to have a poor body condition, as assessed by pectoral muscle mass, and three (12%) had a normal body condition. Seventeen birds (65%) had a multifocal to diffuse, moderate to severe fibrinonecrotic thickening of the crop mucosa (ingluvitis) (Figure 1A,B,C), often with inflammation extending to the underlying connective tissue and muscle. This pathology represented the most frequently reported gross finding. Birds with these crop lesions sometimes also had a fibrinonecrotic hepatitis (Figure 1D) and or splenitis (9 birds, 35%) and several had evidence of moderate to severe, locally extensive small intestinal haemorrhage (6 birds, 23%). Salmonellosis was considered the cause of death in 20 (77%) of the birds examined and was considered an incidental finding in two (8%) birds (a greenfinch and a Eurasian siskin). The role of salmonellosis in the death of four birds was inconclusive from post-mortem examination (a Eurasian siskin, a house sparrow, a wood pigeon and a collared dove); three of these birds were of poor body condition but no lesions typically associated with salmonellosis were found, and one bird was too decomposed to conduct a conclusive post-mortem examination. None of the gull carcases from which *Salmonella* was isolated were subject to a full post-mortem examination.

Table 1: Characteristics of *Salmonella enterica* isolates and their wild bird hosts. Isolates are listed in order of the location at which *Salmonella* infected birds were found or sampled.

Origin	Sample date	Location	Age	Sex	Dead/alive	Serotype	Phage type	PFGE pattern
House sparrow	02/02/05	1	Unknown	female	Alive	Typhimurium 4,12:i	DT56	5
Herring gull	15/09/05	2	Juvenile	unknown	Dead	I Rough: i: 1,2	Unknown	3
House sparrow	26/10/05	3	Unknown	male	Dead	Typhimurium 4,12:i	DT40	6
Greenfinch	22/10/05	4	Adult	male	Dead	Typhimurium 4,12:i	DT40	6
Greenfinch	19/11/05	5	Adult	male	Dead	Typhimurium 4,12:i	DT56	5
Greenfinch	09/01/06	5	Juvenile	male	Dead	Typhimurium 4,12:i	DT56	5
Eurasian siskin	15/01/06	5	Adult	female	Dead	Typhimurium 4,12:i	DT56	5
Goldfinch	03/01/06	6	Juvenile	unknown	Dead	Typhimurium 4,12:i	DT56	5
House sparrow	29/12/05	7	Juvenile	male	Dead	Typhimurium 4,12:i	PT U277	5
House sparrow	09/01/06	8	Unknown	male	Dead	Typhimurium 4,12:i	DT56	5
Eurasian siskin	20/01/06	9	Unknown	male	Dead	Typhimurium 4,12:i	DT56	5
Eurasian siskin	20/01/06	9	Juvenile	male	Dead	Typhimurium 4,12:i	DT56	5
Eurasian siskin	26/01/06	9	Adult	male	Dead	Typhimurium 4,12:i	DT56	5
Eurasian siskin	06/04/06	9	Adult	female	Dead	Typhimurium 4,12:i	DT56	5
House sparrow	18/01/06	10	Unknown	male	Dead	Typhimurium 4,12:i	DT56	5
Greenfinch	01/02/06	11	Juvenile	male	Dead	Typhimurium 4,12:i	DT56	5
Collared dove	27/01/06	12	Adult	female	Dead	Typhimurium 4,12:i	DT56	5
Wood pigeon	05/02/06	13	Unknown	male	Dead	Typhimurium 4,12:i	DT56	5
House sparrow	19/02/06	13	Unknown	male	Dead	Typhimurium 4,12:i	DT56	5
Eurasian siskin	08/02/06	14	Adult	male	Dead	Typhimurium 4,12:i	DT56	5
Greenfinch	09/02/06	15	Juvenile	female	Dead	Typhimurium 4,12:i	DT56	5
Greenfinch	15/10/06	15	Adult	male	Dead	Typhimurium 4,12:i	DT56	5
Greenfinch	10/03/06	16	Juvenile	female	Dead	Typhimurium 4,12:i	DT56	5
Greenfinch	20/02/06	17	Adult	female	Dead	Typhimurium 4,12:i	DT56	5
Eurasian siskin	14/03/06	18	Juvenile	male	Dead	Typhimurium 4,12:i	DT56	5
Eurasian siskin	23/03/06	19	Juvenile	male	Dead	Typhimurium 4,12:i	DT40	4
Greenfinch	28/04/06	20	Adult	male	Dead	Typhimurium 4,12:i	DT56	5
Common starling	16/08/06	21	Unknown	female	Alive	Typhimurium 4,5,12:i	DT41	3
Common starling	16/08/06	21	Unknown	female	Alive	Typhimurium 4,5,12:i	DT41	3
Goldfinch	17/10/06	22	Adult	male	Dead	Typhimurium 4,12:i	DT56	5
Herring gull	02/08/05	23	Juvenile	female	Dead	Seftenberg	NA	1
Black-headed gull	18/08/05	24	Juvenile	unknown	Dead	Newport	NA	2

Antimicrobial susceptibility testing and PCR-virulotyping

Each of the isolates were found to be susceptible to all of the antimicrobials tested, and all contained *prgH*, *sopB*, *invA*, *spiC*, *sifA*, *misL*, *pipD*, *iroN*, *sitC*, *orfL* genes as detected by PCR. The fimbrial associated virulence gene, *pefA*, was absent from most isolates apart from three, two of which were *S.* Typhimurium DT 41 isolates from live starlings caught at the same location and date. The other isolate containing *pefA* was the isolate that could not be characterised by serotyping from a dead herring gull, which was sampled at a different location from, and one year later than, the two live starlings. These three isolates also shared the same unique, pulsed field pattern (Figure 2 – green markers). The *sopE* gene, which has been associated with enteritis and epidemics, particularly affecting humans, was absent from all isolates except the control strain *S.* Typhimurium SL1344.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) revealed six *Xba*I banding patterns, labelled for the purposes of this analysis as 1–6. Each consisted of 13 or 14 DNA fragments, with relative molecular weights ranging from 48.5 kb to 1018.5 kb (Figure 3). Two patterns (1 and 2) were unique to the single isolates of *S.* Senftenberg and *S.* Newport (respectively). Four patterns were identified amongst the *S.* Typhimurium isolates; two (groups 4 and 6) amongst isolates belonging to the phage type DT 40. Both DT 41 isolates clustered in pulsed-field group 3 together with the isolate that could not be identified by serotyping, and DT 56 isolates all clustered in group 5. The *S.* Typhimurium PT U277 isolate also had a group 5 banding pattern. Very high genetic similarity (>90%) was seen within each pulsed-field group. Across all *S.* Typhimurium isolates, analysis of the PFGE banding patterns suggested an overall genetic similarity of 77%, with a genetic similarity of 99% among DT 56 and PT U277 isolates. These *Xba*1 PFGE groups were confirmed by PFGE after digestion with the *Spe*1 restriction enzyme.

Multiple birds were examined from three of 24 locations (Figure 2, Table 1) (<3 month interval between cases at

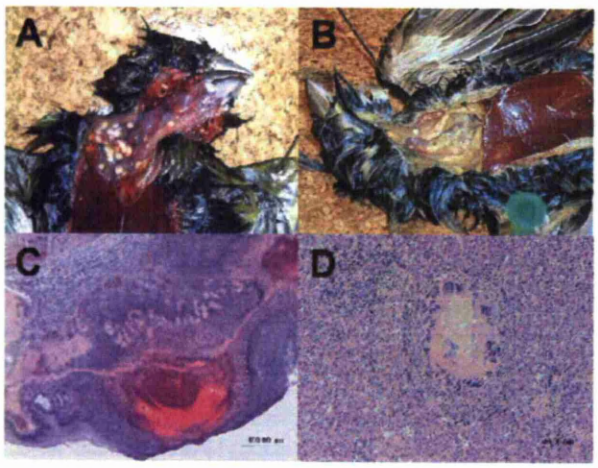

Figure 1
Gross- and histo-pathology associated with *Salmonella* infection in wild passerine species. A: Greenfinch crop: multifocal necrotic ingluvitis. B: Greenfinch crop: diffuse necrotic ingluvitis. C: Greenfinch crop (Haematoxylin and Eosin stain): one central and one peripheral nodule of necrotic crop mucosa and submucosa with haemorrhage and surrounded by zones of infiltrating leucocytes. D: Greenfinch liver (Haematoxylin and Eosin stain): multifocal hepatic necrosis surrounded by a zone of macrophages and multinucleate giant cells which is further surrounded by lymphocytes.

same site). The same phage type and PFGE profile was found in all isolates cultured from the same location.

Cell invasion assays

All *Salmonella* isolates tested showed similar levels of invasiveness for avian HD11 cells, as demonstrated by the bacterial counts performed one hour after infection (Figure 4). In addition, all isolates tested persisted within the cells for 24 hours and in most cases, underwent replication.

Discussion

The data presented here show that salmonellosis of wild, particularly passerine birds, in the north of England during 2005 and 2006 was caused mainly by a narrow range of possibly host-adapted *S.* Typhimurium strains. These strains are generally susceptible to antimicrobials in contrast to many human and livestock-associated strains [12,13] such as *S.* Typhimurium DT 104 [12-14]. They contain a range of genes associated with systemic and enteric disease in birds and mammals, but lack *sopE*, a gene that has been associated with some epidemic strains of *S.* Typhimurium in both humans and food animals. The *sopE* gene has been identified in six of 15 outbreak-associated *S.* Typhimurium phage types, but not in the epidemic multiresistant phage type DT 104, which has been the most common *S.* Typhimurium phage type in cases of human infection in England and Wales since 1990 [14].

The majority of *Salmonella* isolates obtained during this study were of serovar Typhimurium, of which four phage types were identified; DT 40, DT 41, DT 56 and PT U277, with DT 56 being the predominant phage type, widespread spatially and temporally over the north of England (Figure 2). As far as we know, *S.* Typhimurium DT 56 has not been reported previously in wild birds from outside of the UK, unlike phage types DT 40 and PT U277 that have been reported in wild birds from Scandinavia [15]. It is possible that the predomination of phage type DT 56 may result from the fact that sample collection was geographically and temporally limited to the north of England in 2005 and 2006, therefore a single clone may have been the origin of most of the DT 56 isolates. This hypothesis would be supported by the results of the PFGE analysis carried out by this study where all DT 56 isolates showed 99% similarity to each other. The majority of *S.* Typhimurium DT 56 and DT 40 isolates were from members of the *Fringillidae* (greenfinch, goldfinch and siskin) and *Passeridae* (house sparrow) families, which is consistent with the findings in previous studies [3,4,16]. In addition, *S.* Typhimurium DT56 was isolated from a wood pigeon and a collared dove. *S.* Typhimurium DT 41 was isolated from starlings and one herring gull: the findings of this study and others would suggest that DT 41 causes only sporadic mortality in 'garden birds' and is more frequently associated with gull and wildfowl species [4].

S. Typhimurium DTs 40 and 56 were those most often associated with garden bird mortality, as found in previous studies [4,17,18]. Pulsed-field gel electrophoresis demonstrated that the *S.* Typhimurium isolates in this study belonged to a small number of closely related, sometimes clonal, strains. This suggests that *S.* Typhimurium infection in garden birds is maintained within the wild bird population rather than there being repeated infection of wild birds from other sources. However, we cannot state that transmission never occurs between wild birds and livestock or humans, and wild birds should therefore still be treated as a potential source of *Salmonella* infection. Indeed, a number of studies carried out in Norway and New Zealand have shown an epidemiological link between wild passerines and human *Salmonella* outbreaks [11,19].

The *S.* Typhimurium isolates clustered into four main PFGE groups which, with three exceptions, were closely correlated to phage-type. An isolate of PT U277 showed 99% similarity to the DT 56 isolates that clustered together in PFGE group 5. PFGE analysis grouped 3 DT 40 isolates into 2 patterns (groups 4 and 6) that were sepa-

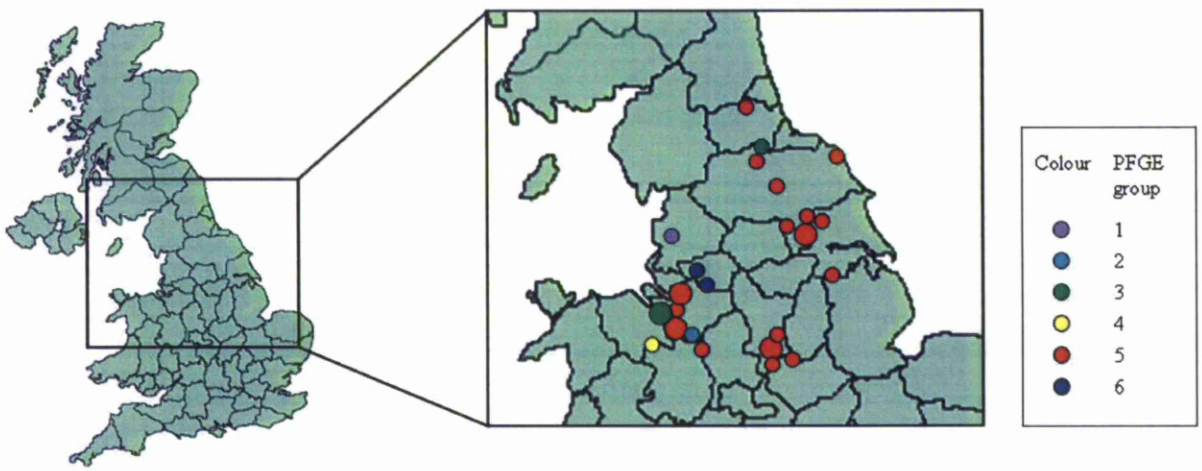

Figure 2
Map of the distribution of birds from which *Salmonella enterica* were isolated. Points on the map are colour coded according to the pulsed-field gel electrophoresis banding patterns obtained using *Xba I* and *Spe I* enzymes. Larger points represent sites from which more than one *Salmonella* infected dead bird was found.

rated geographically (Figure 2). Two DT 41 isolates from live starlings sampled at the same location on the same date shared an identical PFGE pattern with the isolate from a dead herring gull that could not be classified by serotyping, sampled at a different location on a different date. Interestingly, these last three isolates were the only ones to contain the fimbrial associated virulence gene *pefA*.

PFGE group 5 contained the largest number of isolates, including all of the DT56 isolates obtained during this study, demonstrating that these isolates are members of a clonal strain. Alley *et al* [19] reported a similar finding that all DT160 isolates examined by PFGE during an outbreak of salmonellosis in wild passerines and humans in New Zealand were indistinguishable and therefore members of a clonal strain. Due to the small number of isolates phage-typed as DT40 and DT41 in this study, it is not possible to comment on their clonality. *S.* Typhimurium DT 40 is known to affect wild birds, particularly passerine species [4], therefore it is important to investigate variation within this phage type further. Similar work carried out on a national scale over a longer time period would be valuable.

Every isolate was found to possess all of the virulence genes that were screened for apart from *pefA* and *sopE*. The *pefA* gene is located on a virulence plasmid rather than the bacterial chromosome [20]. Such plasmids can be serovar-specific, but it has been found that not all isolates of plasmid-bearing serovars contain these plasmids [21,22]. This may explain the low prevalence of this gene among the *Salmonella* isolates obtained in the present study. The rest of the virulence-associated genes screened for are located on *Salmonella* pathogenicity islands (PAIs) 1–5 [21,23,24], and are known to be associated with adhesion, cell invasion and intra-cellular survival. These genes, particularly those associated with the *Salmonella* pathogenicity island 1 (SPI-1) and *Salmonella* pathogenicity island 2 (SPI-2) type III secretion systems, have been well-characterised for their role in both enteritis and systemic infection in mammalian models. Their roles in avian infection are less clear, though the SPI2 system is a requirement for infection and disease in poultry with the avian specific serovars *S.* Gallinarum and *S.* Pullorum [25,26]. Recently SPI1 and SPI2 have been shown to be involved in both systemic and gastrointestinal tract infection of the chicken by *S.* Typhimurium. The possession of these virulence factors would suggest that the isolates investigated in the current study have the ability to cause systemic and enteric salmonellosis in their hosts. In addition, the ability of all strains tested to invade and persist in avian macrophage-like HD11 cells, would also suggest that they have the potential to cause systemic infection in avian species: mutant strains of *Salmonella* incapable of

Source	Serovar	Phage-type	PFGE group
Herring gull	Seftenberg		1
Black-headed gull	Newport		2
Herring gull	Typhimurium	Unknown	3
Common starling	Typhimurium	DT41	3
Common starling	Typhimurium	DT41	3
Siskin	Typhimurium	DT40	4
Greenfinch	Typhimurium	DT56	5
Greenfinch	Typhimurium	DT56	5
Siskin	Typhimurium	DT56	5
Siskin	Typhimurium	DT56	5
Greenfinch	Typhimurium	DT56	5
House sparrow	Typhimurium	DT56	5
Greenfinch	Typhimurium	DT56	5
Goldfinch	Typhimurium	DT56	5
House sparrow	Typhimurium	PTU277	5
House sparrow	Typhimurium	DT56	5
Greenfinch	Typhimurium	DT56	5
Siskin	Typhimurium	DT56	5
Siskin	Typhimurium	DT56	5
House sparrow	Typhimurium	DT56	5
Greenfinch	Typhimurium	DT56	5
Collared dove	Typhimurium	DT56	5
Siskin	Typhimurium	DT56	5
Wood pigeon	Typhimurium	DT56	5
Siskin	Typhimurium	DT56	5
Greenfinch	Typhimurium	DT56	5
Greenfinch	Typhimurium	DT56	5
House sparrow	Typhimurium	DT56	5
Goldfinch	Typhimurium	DT56	5
Siskin	Typhimurium	DT56	5
F98	Typhimurium	CONTROL	
House sparrow	Typhimurium	DT40	6
Greenfinch	Typhimurium	DT40	6
SL1344	Typhimurium	CONTROL	

Figure 3
Dendrogram showing the genetic similarity (%) between *Salmonella enterica* isolates digested with *Xba I* restriction enzyme.

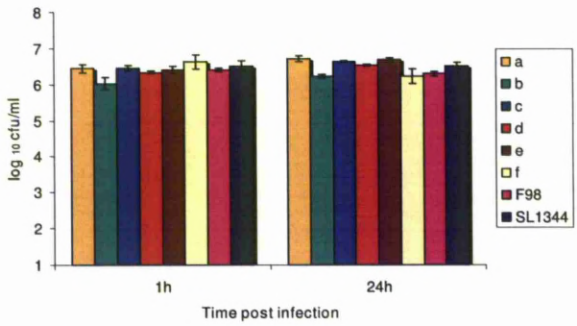

Figure 4
Uptake and persistence of *Salmonella* isolates from wild birds in avian HD11 cells. Viable counts of a sub-sample of *Salmonella enterica* serovar Typhimurium isolates, plus an isolate that could not be characterised by serotyping, expressed as CFU/ml were determined on nutrient agar after the ability of the isolates to invade and persist in avian HD11 cells was assessed using a gentamicin protection assay. Within the sub-sample there were isolates of each phage type and *S.* Typhimurium pulsed field group identified in this study (a – f) plus two control strains (F98 and SL1344).

surviving within macrophages *in vitro* are attenuated for systemic virulence [25,27].

Interestingly, the *Salmonella* isolate obtained from a live house sparrow was of the same virulence genotype as the isolates from dead house sparrows and other species. Skyberg *et al* [21] found that a number of virulence genes could be detected in both healthy and sick poultry, indicating that some virulence genes and associated PAIs may be widespread in salmonellae isolated from both healthy and sick birds. It may be that other factors, such as nutritional, environmental or physiological stressors are also involved in the development of clinical disease and mortality, or it could be that these healthy birds had only very recently become infected and had not had time to develop disease. Experimental studies have shown that gastrointestinal carriage of *Salmonella* occurs in passerines after infection; therefore it is possible that healthy birds could be persistent carriers [28].

None of the isolates examined possessed the *sopE* gene, which has been found to be present in some strains of *S.* Typhimurium associated with epidemic disease in both humans and food animals [29] but not with *S.* Typhimurium DT 104 [24]. This may indicate that wild passerine strains of *S.* Typhimurium are generally not involved in the epidemiology of *S.* Typhimurium infection in humans or livestock in the UK. The absence of antibiotic resistance detected in this study would also support this hypothesis, as many *S.* Typhimurium isolates from human or production animal sources are resistant to at least one antibiotic [12-14]. It is possible that the *S.* Typhimurium strains affecting wild passerines are adapted to, and maintained within, the wild bird population. However, this study examined isolates collected only during 2005 and 2006. It would require a more spatially extensive study carried out over a longer time period to gather sufficient evidence to fully support this hypothesis.

The gross post-mortem findings in 20 out of 26 birds examined were similar to those described in previous studies [4,30-33]. Lesions were most commonly present in the crop of the bird, and it has been suggested that ingluvitis and oesophagitis in passerines with salmonellosis indicates that the crop and oesophagus are predilection sites for bacterial invasion. It is possible that progression of this infection to systemic salmonellosis involving other organs (liver and spleen) may occur if the bird's immune system became further compromised [34]. No gross lesions were noted in two dead birds from which *Salmonella* was isolated, a greenfinch and a Eurasian siskin. In these cases where *Salmonella* appeared as an incidental finding, it is impossible to know if these birds would have succumbed to salmonellosis in the future (had they not been killed by some other cause) or for how long the bird had been infected before death. Studies have shown that chickens can carry *Salmonella enterica* containing virulence genes asymptomatically [21], and this must therefore be a possibility in wild birds also. The only way to determine if this is the case would be through a longitudinal study of individual wild birds.

Conclusion

Molecular characterisation of the *Salmonella* isolates collected from both live and dead wild birds in this study has indicated that wild passerine deaths in northern England associated with salmonellosis during 2005 and 2006 were caused by a small number of closely-related *S.* Typhimurium strains, some of which appear to be clonal. These strains were typically susceptible to antimicrobials, capable of invading and persisting within avian macrophage-like cells and contained a range of virulence factors associated with intra-cellular survival, adhesion and invasiveness. All the isolates tested lacked the *sopE* gene associated with some *S.* Typhimurium disease outbreaks in humans and livestock, which suggests that it is unlikely that these isolates represent a large zoonotic risk. These findings also suggest that *S.* Typhimurium infection in wild passerines is maintained within wild bird populations and may be host-adapted. To investigate this further, similar work would need to be carried out on a national scale over a longer time period.

Table 2: Virulence genes and PCR primers used to test screen *Salmonella* isolates.

Virulence gene	Pathogenicity island	Gene function	Broad action	Primer sequence (5' to 3')
prgH	SPI-1	Type III secretion system apparatus	Invasion of macrophages	F: GCCCGAGCAGCCTGAGAAGTTAGAAA R: TGAAATGAGCGCCCCTTGAGCCAGTC
sopB	SPI-1	Type III secreted effector protein	Invasion of macrophages	F: CGGACCGCCCAGCAACAAAACAAGAAGAAG R: TAGTGATGCCCGTTATGCGTCAGTGTATT
sopE	SPI-1	Type III secreted effector protein	Invasion of macrophages	F: TCAGTTGGAATTGCTGTGGA R: TCCAAAAACAGGAAACCACAC
invA	SPI-1	Type III secretion system apparatus	Invasion of macrophages	F: CTGGCGGTGGGTTTTGTTGTCTTCTCTATT R: AGTTTCTCCCCCTCTTCATGCGTTACCC
sitC	SPI-1	Iron transport	Invasion of macrophages/ iron acquisition	F: CAGTATATGCTCAACGCGATGTGGGTCTCC R: CGGGGCGAAAATAAAGGCTGTGATGAAC
spiC	SPI-2	Type III secretion system	Survival in macrophages	F: CCTGGATAATGACTATTGAT R: AGTTTATGGTGATTGCGTAT
sifA	SPI-2	Type III secreted effector protein	Survival in macrophages	F: TTTGCCGAACGCGCCCCCACACG R: GTTGCCTTTTCTTGCGCTTTCCACCCATCT
misL	SPI-3	Involved in intramacrophage survival	Survival in macrophages	F: GTCGGCGAATGCCGCGAATA R: GCGCTGTTAACGCTAATAGT
orfL	SPI-4	Adhesin/autotransporter	Survival in macrophages/ colonisation	F: GGAGTATCGATAAAGATGTT R: GCGCGTAACGTCAGAATCAA
pipD	SPI-5	Type III secreted effector-associated with SPI-1 system	Enteritis	F: CGGCGATTCATGACTTTGAT R: CGTTATCATTCGGATCGTAA
iroN	NA	Siderophore (iron acquisition)	Associated with iron usage	F: ACTGGCACGGCTCGCTGTCGCTCTAT R: CGCTTTACCGCCGTTCTGCCACTGC
pefA	NA	Fimbriae	Movement	F: GCGCCGCTCAGCCGAACCAG R: CAGCAGAAGCCCAGGAAACAGTG

Methods

Bacterial isolates

Thirty two *Salmonella* isolates were collected from wild birds from a total of 24 sites across northern England (Table 1 and Figure 2) between July 2004 and January 2007. The number of isolates collected from each location is detailed in Table 1. Isolates were obtained from a total of 2100 faecal samples collected from apparently healthy wild birds caught primarily for ringing (85.5% of samples), dead birds submitted to a local wildlife hospital (8.5% of samples), and dead birds collected from members of the public through the Garden Bird Health Initiative (6% of samples) [35]. Where possible, a full post-mortem examination was carried out on dead birds.

All faecal samples collected were processed in the laboratory by the author within 24 hours of sample collection. *Salmonella* bacteria were isolated using a routine method [36]: briefly faecal samples were enriched aerobically in peptone buffer at 37°C for 24 hours followed by incubation in Rappaport-Vassiliadis broth at 42°C in aerobic conditions again for 24 hours, before inoculation onto Rappaport-Vassiliadis agar and incubation for 24 hours at 37°C. Colonies suspected of being salmonella were confirmed using an API20E kit (Biomérieux, l'Etoile, France) according to the manufacturer's instructions. Isolates were serotyped by performing slide agglutination tests with *Salmonella* O and H group antisera (Pro-Lab, Neston, UK). All *S.* Typhimurium isolates were phage-typed as previously described [37,38].

Antimicrobial susceptibility testing

Salmonella Typhimurium isolates were tested for antimicrobial susceptibility by disk diffusion on iso-sensitest agar (LabM, Bury, UK) using British Society for Antimicrobial Chemotherapy (BSAC) guidelines for testing enterobacteriaceae [39]. Isolates were tested for susceptibility to amikacin (AMK), amoxicillin/clavulanic acid (AMX), ampicillin (AMP), cefpodoxime (CPD), cefoxitin (FOX), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), oxytetracycline (OTC) and trimethoprim/sulfamethoxazole (SXT).

PCR virulotyping

Crude DNA extracts were prepared for each isolate by boiling in water for 20 minutes, and screened by polymerase chain reaction (PCR) for the presence of several genes thought to be associated with virulence [21,23,24] (Table 2). The reaction mixtures and cycling conditions were the same for all reactions: 1.25 units Taq DNA polymerase (ABgene, Epsom, UK); 75 mM Tris-HCl (pH 8.8 at 25°C); 20 mM $(NH_4)_2SO_4$; 2.5 mM $MgCl_2$; 0.01% (v/v) Tween 20; 0.2 mM each of dATP, dCTP, dGTP and dTTP; 4 mM forward and reverse primers; with 1 microlitre of the DNA template in a final reaction volume of 25 μl. DNA amplification was carried out in a Thermo Hybaid thermocycler

(ABgene, Epsom, UK) with an initial denaturation step at 94°C for 3 minutes followed by 30 cycles of amplification (denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute) with a final extension step at 72°C for 5 minutes, followed by a holding temperature of 10°C. The PCR products were separated by gel electrophoresis in a 2% agarose gel in Tris-acteate buffer. Ethidium bromide was added to the agarose, and the gel was visualized under ultra-violet light. Amplicon size was determined by comparison with ΦX174 Hae III Digest DNA marker (ABgene, Epsom, UK). Two control *S.* Typhimurium strains were used: *S.* Typhimurium SL1344 and *S.* Typhimurium F98 [40,41].

Pulsed-field gel electrophoresis (PFGE)

Block preparation and PFGE were performed according to the Standardised PulseNet Rapid *E. coli* PFGE method with slight modifications [42]. Genomic DNA from *Salmonella* isolates was digested using 50 U per sample *XbaI* (Promega, Southampton, UK) or 15 U per sample *SpeI* (Promega) enzymes for 2 hours or 22 hours respectively. Macro-restriction digested fragments were separated on a 1% agarose gel (pulsed-field certified, BioRad Laboratories, Hertfordshire, UK), at 210 volts for 19 hours at 14°C on a CHEF DRIII system (Bio-Rad Laboratories). Pulse times were ramped from 2.2–54.2 seconds and a reorientation angle of 120° was applied. Bacteriophage λ DNA concatemers (Bio-Laboratory) embedded in 1% LMP agarose were used as molecular weight markers, and *S.* Typhimurium SL1344 and *S.* Typhimurium F98 were again used as controls. Gels were stained for 20 minutes with 1% ethidium bromide solution and visualised using UV light. BioNumerics version 4.0 (Applied Maths BVBA, Sint-Martens-Latem, Belgium) software was used for image analysis. A percentage similarity between pulse-field banding patterns was computed according to the Dice similarity coefficient method with a 2% tolerance window, and a dendrogram was constructed using the UPGMA (unweighted pair group method with averages).

Cell invasion assays

Six isolates of *S.* Typhimurium, including isolates from both live and dead birds plus a representative sample of each *S.* Typhimurium PFGE pattern and phage type identified, were tested for their ability to invade and persist in avian macrophage-like HD11 cells using a gentamicin protection assay as previously described by Jones *et al* [25]. Chicken macrophage-like cell line HD11 was used for the assays [43]. For bacterial counts, a modified Miles-Misra method was used to estimate the number of bacteria per millilitre of macrophage cells one hour post-infection and 24 hours post-infection. Each assay was repeated 3 times for each bacterial isolate and an average bacterial count is presented in the results.

Authors' contributions

LH performed post-mortem examinations and *Salmonella* isolations. LH and SS conducted the majority of the experimental work and co-wrote the manuscript. PW designed the virulotyping, PFGE and cell invasion experiments, helped design the study and co-wrote the manuscript. HB conducted the initial pilot study. AHW helped conduct and analysed the PFGE data. NW designed and helped conduct the antimicrobial sensitivity testing. MB helped design and analyse the study and co-wrote the manuscript. EDP performed the phage typing and contributed to the writing of the manuscript. BL and AC coordinated the Garden Bird Health Initiative through which a number of the samples were collected, and contributed towards the study design. JC performed post-mortem examinations and their analysis, helped in the study design and co-wrote the manuscript. All authors read and approved the final manuscript.

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References

1. Wilson JE, MacDonald JW: **Salmonella infection in wild birds.** *British Veterinary Journal* 1967, **123**:212-219.
2. Goodchild WM, Tucker JF: **Salmonellae in British wild birds and their transfer to domestic fowl.** *British Veterinary Journal* 1968, **124**:95-100.
3. Refsum R, Handeland K, Baggesen DL, Holstad G, Kapperud G: **Salmonellae in avian wildlife in Norway from 1969 to 2000.** *Applied and Environmental Microbiology* 2002, **68**:5595-5599.
4. Pennycott T, Park A, Mather HA: **Isolation of different serovars of Salmonella enterica from wild birds in Great Britain between 1995 and 2003.** *The Veterinary Record* 2006, **158**:817-820.
5. Girwood RWA, Fricker CR, Munro D: **The incidence and significance of Salmonella carriage by gulls (Larus spp.) in Scotland.** *Journal of Hygiene* 1985, **95**:229-241.
6. Monaghan P, Shedden CB, Ensor K, Fricker CR, Girwood RWA: **Salmonella carriage by herring gulls in the Clyde area of Scotland in relation to their feeding ecology.** *Journal of Applied Ecology* 1985, **22**:669-680.
7. Quessy S, Messier S: **Prevalence of Salmonella spp, Campylobacter spp and Listeria spp in ring-billed gulls (Larus delawarensis).** *Journal of Wildlife Diseases* 1992, **28**:526-531.
8. Morishita TY, Aye PP, Ley EC, Harr BS: **Survey of pathogens and blood parasites in free-living passerines.** *Avian Diseases* 1999, **43**:549-552.
9. Reilly WJ, Forbes GI, Patterson GM, Sharp JCM: **Human and animal salmonellosis in Scotland associated with environmental contamination, 1973-74.** *The Veterinary Record* 1981, **108**:553-555.
10. Coulson JC, Butterfield J, Thomas C: **The herring gull Larus argentatus as a likely transmitting agent of Salmonella montevideo to sheep and cattle.** *Journal of Hygiene* 1983, **91**:437-443.
11. Kapperud G, Stenwig H, Lassen J: **Epidemiology of Salmonella typhimurium O-12 infection in Norway. Evidence of trans-**

mission from an avian wildlife reservoir. *American Journal of Epidemiology* 1998, **147**:774-782.
12. Cooke FJ, Wain J: **Antibiotic resistance in Salmonella infections.** In *Salmonella Infections: Clinical, Immunological and Molecular Aspects* Edited by: Mastroeni P, Maskell D. Cambridge , Cambridge University Press; 2006:25-56.
13. Poppe C, Ziebell K, Martin L, Allen K: **Diversity in antimicrobial resistance and other characteristics among Salmonella Typhimurium DT104 isolates.** *Microbial Drug Resistance* 2002, **8**:107-122.
14. Threlfall EJ, Teale CJ, Ward LR, Davies RH, Skinner JA, Graham A, Cassar C, Speed K: **A comparison of antimicrobial susceptibilities in non-typhoidal salmonellas from humans and food animals in England and Wales.** *Microbial Drug Resistance* 2003, **9**:183-189.
15. Refsum R, Heir E, Kapperud G, Vardund T, Holstad G: **Molecular epidemiology of Salmonella enterica serovar Typhimurium isolates detected by pulsed-field gel electrophoresis: comparison of isolates from avian wildlife, domestic animals, and the environment in Norway.** *Applied and Environmental Microbiology* 2002, **68(11)**:5600-5606.
16. DEFRA, OIE: **Wildlife diseases in the UK.** [http://www.defra.gov.uk/corporate/vla/science/documents/science-end-oie05.pdf].
17. Pennycott T, Cinderey RN, Park A, Mather HA, Foster G: **Salmonella enterica subspecies enterica serotype Typhimurium and Escherichia coli 086 in wild birds at two garden sites in south-west Scotland.** *The Veterinary Record* 2002, **151**:563-567.
18. Duff JP: **Wildlife diseases in the UK 2002.** *Veterinary Laboratories Agency Report* 2003:18-24.
19. Alley MR, Connolly JH, Fenwick SG, Mackereth GF, Leyland MJ, Rogers LE, Haycock M, Nicol C, Reed CEM: **An epidemic of salmonellosis caused by Salmonella Typhimurium DT160 in wild birds and humans in New Zealand.** *New Zealand Veterinary Journal* 2002, **50(5)**:170-176.
20. Gulig PA, Danbara H, Guiney DG, Lax AJ, Norel F, Rhen M: **Molecular analysis of spv virulence genes of the Salmonella virulence plasmids.** *Molecular Microbiology* 1993, **7**:825-830.
21. Skyberg JA, Logue CM, Nolan LK: **Virulence genotyping of Salmonella spp. with multiplex PCR.** *Avian Diseases* 2006, **50**:77-81.
22. Rotger R, Casadesus J: **The virulence plasmids of Salmonella.** *International Microbiology* 1999, **2**:177-184.
23. Gassama-Sow A, Wane AA, Canu NA, Uzzau S, Aidara-Kane A, Rubino S: **Characterization of virulence factors in the newly described Salmonella enterica serotype Keurmassar emerging in Senegal (sub-Saharan Africa).** *Epidemiology and Infection* 2006, **134(4)**:741-743.
24. Hopkins KL, Threlfall EJ: **Frequency and polymorphism of SopE in isolates of Salmonella enterica belonging to the ten most prevalent serotypes in England and Wales.** *Journal of Medical Microbiology* 2004, **53**:539-543.
25. Jones MA, Wigley P, Page KL, Hulme SD, Barrow PA: **Salmonella enterica serovar Gallinarum requires the Salmonella pathogenicity island 2 type III secretion system but not the Salmonella pathogenicity island 1 type III secretion system for virulence in chickens.** *Infection and Immunity* 2001, **69(9)**:5471-5476.
26. Wigley P, Jones MA, Barrow PA: **Salmonella enterica serovar Pullorum requires the Salmonella pathogenicity island 2 type III secretion system for virulence and carriage in the chicken.** *Avian Pathology* 2002, **31(5)**:501-506.
27. Jones MA, Hulme SD, Barrow PA, Wigley P: **The Salmonella pathogenicity island 1 and Salmonella pathogenicity island 2 type III secretion systems play a major role in pathogenesis of systemic disease and gastrointestinal tract colonization of Salmonella enterica serovar Typhimurium in the chicken.** *Avian Pathology* 2007, **36(3)**:199-203.
28. Connolly JH, Alley MR, Dutton GJ, Rogers LE: **Infectivity and persistence of an outbreak strain of Salmonella enterica serotype DT160 for house sparrows (Passer domesticus) in New Zealand.** *New Zealand Veterinary Journal* 2006, **54(6)**:329-332.
29. Mirold S, Rabsch W, Rohde M, Stender S, Tschape H, Russmann H, Igwe E, Hardt WD: **Isolation of a temperate bacteriophage encoding the type III effector protein SopE from an epidemic Salmonella typhimurium strain.** *Proceedings of the National Academy of Sciences of the United States of America* 1999, **96(17)**:9845-9850.
30. Pennycott T, Ross HM, McLaren IM, Park A, Hopkins GF, Foster G: **Causes of death of wild birds of the family Fringillidae in Britain.** *The Veterinary Record* 1998, **143(6)**:155-158.
31. Refsum R, Vikoren T, Handeland K, Kapperud G, Holstad G: **Epidemiological and pathological aspects of Salmonella Typhimurium infection in passerine birds in Norway.** *Journal of Wildlife Diseases* 2003, **39(1)**:64-72.
32. MacDonald JW, Brown DD: **Salmonella infection in wild birds in Britain.** *The Veterinary Record* 1974, **94**:321-322.
33. Daoust PY, Busby DG, Ferns L, Goltz J, McBurney S, Poppe C, Whitney H: **Salmonellosis in songbirds in the Canadian Atlantic Provinces during winter-summer 1997-98.** *Canadian Veterinary Journal* 2000, **41**:54-59.
34. Tizard I: **Salmonellosis in wild birds.** *Seminars in Avian and Exotic Pet Medicine* 2004, **13(2)**:50-66.
35. Cunningham AA, Lawson B, Bennett M, Chantrey J, Kirkwood J, Pennycott T, Simpson V: **Garden bird health.** *The Veterinary Record* 2005, **156(20)**:656.
36. OIE: **Salmonellosis.** [http://www.oie.int/eng/normes/mmanual/A_00129.htm].
37. Callow BR: **A new phage-typing scheme for Salmonella Typhimurium.** *Journal of Hygiene* 1959, **57**:346-359.
38. Anderson ES, Ward LR, de Saxe MJ, de Sa JDH: **Bacteriophage-typing designations of Salmonella Typhimurium.** *Journal of Hygiene* 1977, **78(2)**:297-300.
39. Andrews J: **British Society of Antimicrobial Chemotherapy (BSAC) methods for antimicrobial susceptibility testing.** [http://www.bsac.org.uk/_db/_documents/version_6.1.pdf].
40. Sanger I: **Salmonella spp. comparative sequencing.** [http://www.sanger.ac.uk/Projects/Salmonella/].
41. Smith HW, Tucker JF: **The effect of antibiotic therapy on the faecal excretion of Salmonella typhimurium by experimentally infected chickens.** *Journal of Hygiene (London)* 1975, **75**:275-292.
42. Centres for Disease Control and Prevention: **One-day (24-28 h) standardized laboratory protocol for molecular subtyping of Escherichia coli 0157:H7, non-typhoidal Salmonella serotypes, and Shigella sonnei by pulsed field gel electrophoresis (PFGE).** [http://www.cdc.gov/PULSENET/protocols/ecoli_salmonella_shigella_protocols.pdf].
43. Beug H, vonKirchbach A, Doderlein G, Conscience JF, Graf T: **Chicken hemapoietic cells transformed by seven strains of defective avian leukemia viruses display three distinct phenotypes of differentiation.** *Cell* 1979, **18**:375-390.

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Emerging Infectious Disease Leads to Rapid Population Declines of Common British Birds

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Abstract

Emerging infectious diseases are increasingly cited as threats to wildlife, livestock and humans alike. They can threaten geographically isolated or critically endangered wildlife populations; however, relatively few studies have clearly demonstrated the extent to which emerging diseases can impact populations of common wildlife species. Here, we report the impact of an emerging protozoal disease on British populations of greenfinch *Carduelis chloris* and chaffinch *Fringilla coelebs*, two of the most common birds in Britain. Morphological and molecular analyses showed this to be due to *Trichomonas gallinae*. Trichomonosis emerged as a novel fatal disease of finches in Britain in 2005 and rapidly became epidemic within greenfinch, and to a lesser extent chaffinch, populations in 2006. By 2007, breeding populations of greenfinches and chaffinches in the geographic region of highest disease incidence had decreased by 35% and 21% respectively, representing mortality in excess of half a million birds. In contrast, declines were less pronounced or absent in these species in regions where the disease was found in intermediate or low incidence. Also, populations of dunnock *Prunella modularis*, which similarly feeds in gardens, but in which *T. gallinae* was rarely recorded, did not decline. This is the first trichomonosis epidemic reported in the scientific literature to negatively impact populations of free-ranging non-columbiform species, and such levels of mortality and decline due to an emerging infectious disease are unprecedented in British wild bird populations. This disease emergence event demonstrates the potential for a protozoan parasite to jump avian host taxonomic groups with dramatic effect over a short time period.

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Introduction

Emerging infectious diseases (EIDs) are increasingly cited as threats to wildlife, livestock and humans alike [1] and can be a major threat to geographically isolated or critically endangered wild bird populations [2,3]. Parasites are integral components of healthy ecosystems, but while impacts on individuals are well recognised [e.g. 4,5] consequences at the population level are poorly understood. Assessing the population impacts of disease, particularly those caused by emerging pathogens, within wildlife populations is problematic because little is known of the background species complement of their parasites and because the detection and diagnosis of disease in most wildlife species is challenging. Also, there usually is a paucity of host population data before and after disease emergence. Consequently, documented population declines of common or widespread avian populations due to infectious disease are rare [6,7,8]. Here we combine systematic large-scale monitoring schemes to quantify the incidence of an emerging disease in three widespread passerine bird species and its population impacts.

Trichomonas gallinae is a common protozoan parasite of pigeons (Columbiformes) which principally infects the upper alimentary tract where it can cause the disease, necrotic ingluvitis [9]. Epizootic mortality in columbiform species has been previously reported [9] and the parasite infrequently infects other avian taxa

such as birds of prey and songbirds [10,11]. Trichomonosis has been postulated to be a factor contributing to the extinction of the passenger pigeon *Ectopistes migratorius* [12] and has been shown to be a significant cause of nestling mortality in the island-endemic pink pigeon *Nesoenas mayeri* [3] and in the Iberian Peninsula population of the Bonelli's eagle *Hieraaetus fasciatus* [13,14]. Both of these small populations are considered to be endangered and, for both, mortality due to trichomonosis has been highlighted as a conservation concern.

Opportunistic surveillance of bird deaths in Britain since 2000 has shown a seasonal pattern in finch mortality driven primarily by low-levels of salmonellosis (Fig. 1, [15]). In autumn 2005, the number of unsolicited reports of bird mortality increased markedly and early investigations identified infection with a trichomonad parasite [16,17]. The geographic spread of these reports was uneven and here we take advantage of long-term volunteer monitoring of garden bird occurrence to (i) quantify disease incidence in three species of common garden bird: greenfinch *Carduelis chloris*, chaffinch *Fringilla coelebs* and dunnock *Prunella modularis*; (ii) demonstrate a spatially contemporaneous decline in the occurrence of frequently affected bird species in gardens; and (iii) combine this with national monitoring of bird abundance to show that this decline was followed by significant reductions in regional breeding populations. Public reporting of wild bird carcasses has been utilised as a surveillance tool for West Nile virus and Usutu virus elsewhere [18,19] and volunteer networks were successfully instituted in North America to elicit reports of diseased birds in order to characterise the spread of mycoplasmal conjunctivitis in the house finch *Carpodacus mexicanus* [20,21]. This is the first time, however, as far as we are aware, that such quantitative monitoring of disease incidence and its population impact has been undertaken using established survey networks.

Results

Identification of the disease epidemic

Opportunistic monitoring of garden bird mortality by the Royal Society for the Protection of Birds (RSPB) between 2001 and 2004 showed an annual seasonal peak in mid-winter (Dec/Jan), with 37–76% of reports per annum occurring in these two months (Fig. 1, K. Peck, *Unpublished data*); post mortem examinations indicated that this seasonal peak was largely due to salmonellosis in Fringillidae and Passeridae species ([15] Kirkwood, Cunningham and Simpson, *Unpublished data*). Between January 1st 2000 and December 31st 2004, we examined 750 garden birds post mortem, of which 67% of greenfinch deaths (168/252 birds) were due to salmonellosis and no cases of finch trichomonosis were confirmed.

Following the index case of trichomonosis in a British finch in April 2005, small numbers of finch mortality incidents were reported throughout 2005, unusually peaking during September to November [17]. In summer 2006, the number of finch, particularly greenfinch, mortality reports increased dramatically with a total of 1054 trichomonosis incidents recorded (according to our incident definition – see below), involving c. 6300 dead greenfinches and chaffinches combined, between 1 April 2006 and 30 September 2006. This comprised 50% of all reported incidents of garden bird morbidity and mortality during this period and compares with 84 incidents of trichomonosis for the same time period in 2005 and sporadic cases (none of which involved finches) in previous years. These reports from the public were unsolicited, not in response to a direct appeal and occurred prior to media coverage of the EID. Sick and dead birds at affected sites were typically observed in close vicinity to garden bird feeding stations and exhibited non-specific signs of malaise, for example lethargy and fluffed-up plumage, frequently in combination with dysphagia.

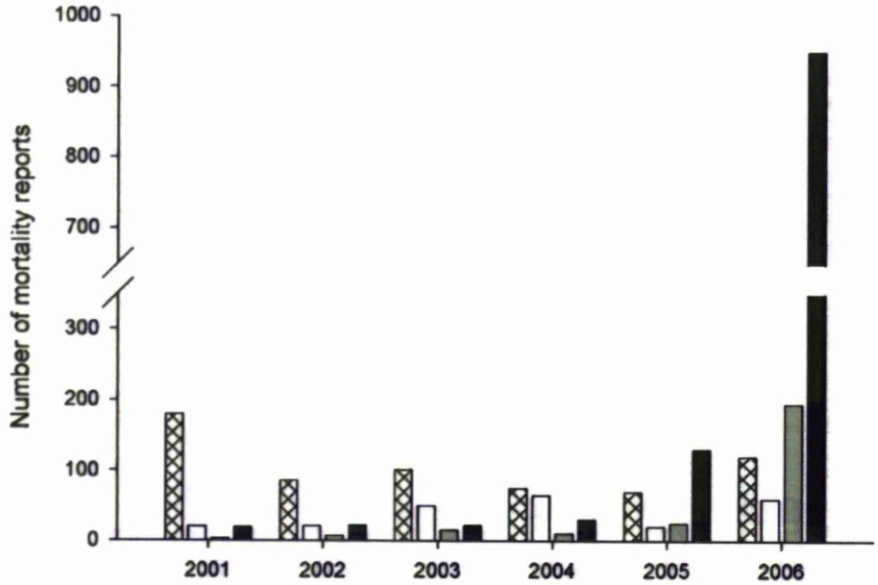

Figure 1. Seasonal incidence of opportunistic reports in all garden bird mortality 2001–2006. Hatched bars – winter (Dec-Feb), open bars – spring (March–May), stippled bars – summer (June–Aug) and black bars - autumn (Sept-Nov). Note break in axis indicating an unprecedented level of reporting in autumn 2006.
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Identification of the disease organism

Necrotic ingluvitis, typically extending through the full thickness of the oesophageal wall and often involving adjacent connective tissue, was diagnosed through post-mortem examination (Fig. 2a) and confirmed as trichomonosis, according to our case definition (see below), in 70 of 125 greenfinches, and in 18 of 76 chaffinches examined between 1 April 2006 and 30 September 2006. These diagnoses were reached on the basis of *T. gallinae* culture alone in 17 birds, nested PCR amplification alone in 58 birds, and a combination of parasite culture and nested PCR in 13 birds. All confirmed trichomonosis cases were negative for *Salmonella* sp. on culture. In addition, 20 of the 125 greenfinches and 29 of the 76 chaffinches were suspected to have died of trichomonosis as these birds had necrotic ingluvitis which was negative for *Salmonella* sp. Trichomonosis in these cases, however, was not confirmed using nested PCR or culture.

Of 241 finch carcasses examined during the period 1 April 2006 to 30 September 2006, 179 were diagnosed as having died of infectious disease. Of these 179 birds, trichomonosis accounted for 144 (80%) of the deaths (90 greenfinches, 47 chaffinches and 7 birds from four other finch species), *E.coli* serotype O86 (the second most common infectious cause of death and which is associated with enteritis with no necrotic ingluvitis) was diagnosed in 24 (13%), while salmonellosis was confirmed in only 4 (2%).

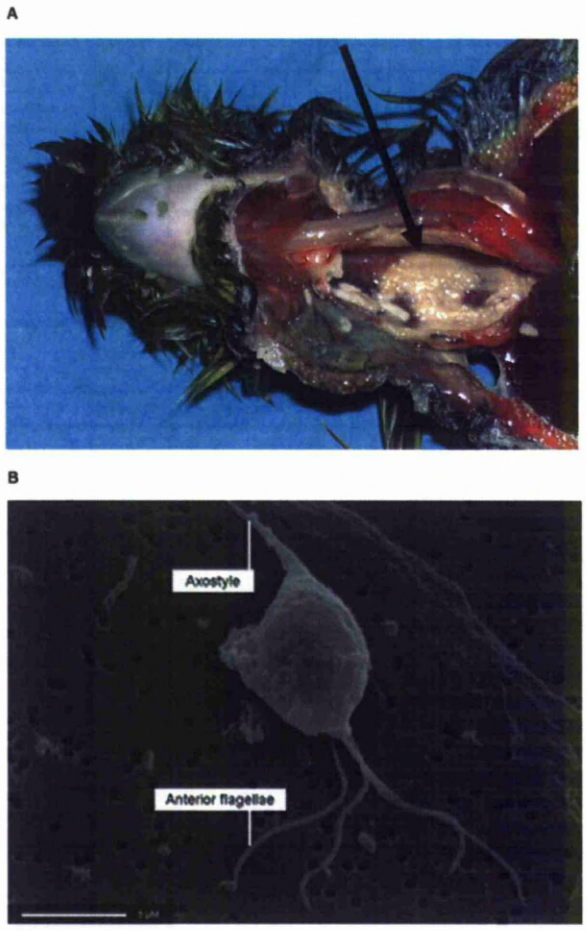

Figure 2. Necrotic ingluvitis lesions and trichomonad parasite morphology. (a) Necrotic ingluvitis lesions (arrow) with a characteristic yellow caseous appearance in a greenfinch caused by *Trichomonas gallinae* infection. (b) Morphology of the greenfinch trichomonad parasite. Scanning electron micrograph. Arrows indicate anterior flagella and axostyle.
doi:10.1371/journal.pone.0012215.g002

Fringillidae species accounted for 84% (292/347) of trichomonosis cases diagnosed in 2005 and 2006, including greenfinch (173 cases), chaffinch (106 cases) and four other finch species (5 bullfinch *Pyrrhula pyrrhula*, 4 goldfinch *Carduelis carduelis*, 2 brambling *Fringilla montifringilla* and 2 siskin *Carduelis spinus*). Columbidae species accounted for 11% (37/347) of cases (20 collared dove *Streptopelia decaocto* and 17 wood pigeon *Columba palumbus*). The only other species in which the disease was diagnosed were house sparrow *Passer domesticus* (9 cases), yellowhammer *Emberiza citrinella* (4 cases), dunnock (3 cases) and great tit *Parus major* (2 cases). Concurrent soiling of the beak and facial plumage with food and saliva was frequently present in affected finches and such birds were typically thin or emaciated. Histopathological examination of the crop confirmed focally extensive moderate to severe mucosal ulceration and submucosal necrosis with infiltration by moderate numbers of degenerate and viable heterophils, lymphocytes and macrophages. Superficially, there was often a layer of necrotic crop epithelial tissue within which groups of 10–20 μm diameter round cells (consistent with protozoal organisms) and numerous clusters of mixed bacterial colonies were seen. Autolysis of the alimentary tract precluded meaningful histological examination in many cases; consequently histopathology was not used as a routine diagnostic test for confirmation of trichomonosis.

Giemsa-stained parasite culture preparations revealed a variable morphology (body dimensions range 8–11×4–5 μm) typical of a trichomonad parasite with a single nucleus and axostyle, anterior flagella and an undulating membrane. Scanning and transmission electron microscopy (Fig. 2b) confirmed the presence of a parasite with plastic pyriform morphology and four anterior flagella that typically exited the body together in pairs. A prominent undulating membrane, with no free posterior trailing flagellum, was present.

Amplification of the ITS1/5.8S/ITS2 ribosomal region was performed on DNA extracted from oesophageal lesions from nine greenfinches and nine chaffinches (submitted from 18 disparate sites across 13 counties covering England, Wales and Scotland) that died of trichomonosis in 2005 or 2006. An identical consensus sequence of 214 nucleotides was identified for all PCR products (Fig. 3a, Genbank accession numbers GQ150752 and GQ150753) from the finch samples examined. National Centre for Biotechnology Information (NCBI) BLAST search identified that the consensus finch sequence matched four Genbank entries with100% sequence identity with 100% query coverage, all of which were for *Trichomonas gallinae* (EU215369 (multiple columbid and hawk species from the USA), EU290649 (house finch *Carpodacus mexicanus*/corvid species from the USA), EF208019 (Mauritian columbid species), AY349182 (*T. gallinae* strain g7)). Thus the organism infecting the British finches was identified as *T. gallinae*.

Sequencing of second stage products of a nested PCR for the detection of trichomonads from oesophageal lesion extracts from seven greenfinches and one chaffinch examined in 2005 or 2006 with trichomonosis identified a consensus sequence of 149 nucleotides in all eight cases (Fig. 3b, Genbank accession no GQ214405). NCBI BLAST identified the consensus finch sequence as a match for four *T. gallinae* Genbank entries (EU215372.1 (Cooper's hawk *Accipiter cooperii*), EU215373.1 (rock

(a)

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A 1.   TAACTTCATCAAAAAATCAAGTCTCTAAGCAACGGATGTCTTGGCTCCTC
B 1.   TAACTTCATCAAAAA-TCAAGTCTCTAAGCAACGGATGTCTTGGCTCCTC

A 51.  ACACGATGAAGAACGTGGCATAATGTGTTAAGTAACCGGAGTTGCAAACA
B 51.  ACACGATGAAGAACGTGGCATAATGTGTTAAGTAACCGGAGTTGCAAACA

A 101. TCATGACAGGTTAATCTTTGAATGCAAATTGCGCTTACCCGGCTTCGGCC
B 101. TCATGACAGGTTAATCTTTGAATGCAAATTGCGCTTACCCGACTTCGGTC

A 151  GAGGAGCATGCGTGTAACAGTACAACATAATTTATAATAATTCTTATTCT
B 151. GAGGAGCATGCGTGTAACAGTACAACATAATTTATAATAATTCTTATTCT

A 201. ACGCGAATAAGCAA
B 201. ACGCGAATAAGCA
```

(b)

```
1.     GGCCGCGCTACTCTTATAATCCCTAACGTAGTTGGGATTGACGTTTGTAA
51.    TCAGCGTCATGAACCAGGAATCCCTTGTAAATGTGTGTCAACAACGCACG
101.   TTGAATACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGAT
```

Figure 3. Sequence data from British finch trichomonad samples. (a) Nucleotide sequence (214 nucleotides) from amplification and sequencing of the ITS1/5.8S/ITS2 ribosomal region using TFR1 and TFR2 primers from (A) Consensus sequence from British finch (Genbank GQ150752 and GQ150753) trichomonad samples and (B) *Trichomonas gallinae* (Rivolta) Stabler (ATCC® Number 30230 TM). (b) Nucleotide sequence (149 nucleotides) from nested PCR with trichomonad SSU rRNA primers followed by TN3 and TN4 nested primers (Genbank GQ214405).
doi:10.1371/journal.pone.0012215.g003

pigeon *Columba livia*), EU215374.1 (collared dove), and EU-215375.1 (broad-winged hawk *Buteo platypterus*); all from the USA) with 100% sequence identity and 100% query coverage and five *Trichomonas* sp. reports with 100% sequence identity and 97% query coverage. Multiple *Tetratrichomonas gallinarum* and *Tetratrichomonas* sp. reports had 98% or less sequence identity with 100% query coverage. The specificity of the nested PCR for a range of species within the Trichomonadidae was not assessed in this study, therefore this technique cannot currently be used in isolation as a diagnostic test for *T. gallinae*. However, use of the nested PCR appears robust within the case definition for finch trichomonosis that we have employed.

Geographical and temporal distribution of the epidemic

Rates of opportunistic reports of trichomonosis (identified according to our incident definition) varied greatly among counties, with rates in excess of 0.20 incidents per thousand households (ipth) found in Gloucestershire, Powys and Warwickshire. We used the opportunistic reports to define regions of High, Intermediate and Low disease incidence (Fig. 4). Overall, the average reporting rate was 0.037 ipth but this varied markedly among the regions (aggregate average for the High region: 0.109 ipth; Intermediate region: 0.056 ipth; Low region: 0.003 ipth, $\chi^2 = 650.5$, d.f. = 2, $p<0.001$). As these rates may have been influenced by local publicity, we quantified incidence in a network of garden sites where diseased birds were searched for systematically according to a defined protocol throughout the study period. Trichomonosis (according to our incident definition) was identified at 39 sites (5.2%) but the incidence varied spatially in a manner similar to the opportunistic reports, with 10.4% (n = 115) of participants in the High region reporting an incident, 7.8% (n = 154) in the Intermediate region and 0.0% (n = 164) in the Low region ($\chi^2 = 16.4$, d.f. = 2, $p<0.005$).

Changes in bird abundance

The weekly reporting rates of greenfinch occurrence in all gardens contributing to the British Trust for Ornithology's (BTO) Garden BirdWatch survey [22] show a seasonal pattern, with more gardens reporting birds in spring and fewer in the autumn (Fig. 5a). There was a significant difference in seasonal pattern of occurrence between 2005 (which was very similar to previous years, Fig. 5b) and 2006, with markedly fewer gardens reporting greenfinches from early August (week 32) onwards (Fig. 5c, $F_{9,23} = 24.23$, $p<0.0001$). Analysis of a subset of these sites which recorded counts of individual birds (rather than presence) showed a similar reduction in mean abundance in gardens reporting greenfinches in the latter half of 2006.

The reporting rate for greenfinches in the following spring (2007) was significantly reduced in the area of High trichomonosis-associated mortality ($\beta = -1.32 \pm 0.12$, $p<0.001$), but less so in the region with Intermediate ($\beta = -0.77 \pm 0.15$, $p<0.001$) or Low mortality levels ($\beta = -0.53 \pm 0.08$, $p<0.01$) (Fig. 6). Reductions in occurrence of chaffinch ($\beta = -0.53 \pm 0.12$, $p = 0.02$) and dunnock ($\beta = -0.25 \pm 0.12$, $p = 0.04$) in the region of High trichomonosis-associated mortality were lower, and there were no significant reductions in occurrence of either species in the regions of Intermediate or Low incidence.

These observed reductions in occurrence were reflected in changes in the size of wider regional breeding populations obtained from the independently-derived Breeding Bird Survey (BBS) [23]. The decline in relative abundance of greenfinches on BBS squares was significantly greater (35%) in the region of High

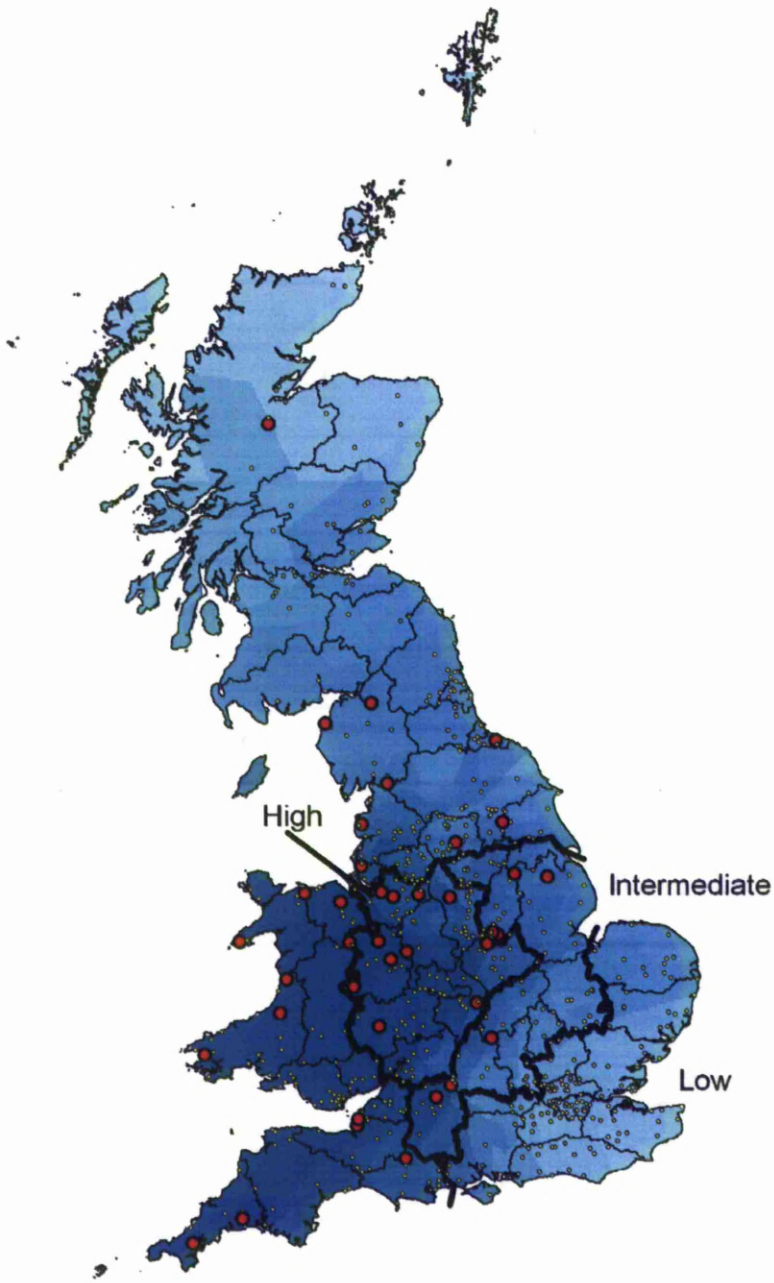

Figure 4. Distribution of finch trichomonosis incidents in 2006. Gardens reporting at least one incident of finch trichomonosis (large red dots) and all other sites (small yellow dots) contributing to the systematic survey. The shading indicates relative incidence of trichomonosis recorded by the opportunistic survey (incidents per thousand households for each county interpolated from county centroids). The heavy lines delineate areas of High, Intermediate and Low incidence, based on the opportunistic survey data.
doi:10.1371/journal.pone.0012215.g004

trichomonosis-associated mortality than in either of the other two regions (Table 1). This annual change was much more marked than any seen in the previous ten years (Fig. 7). In accordance with our predictions, the number of breeding chaffinches declined significantly (21%) in the High incidence region but not in the other two areas; abundance of breeding dunnocks did not decline in any region.

Discussion

We diagnosed trichomonosis as an emerging and widespread cause of death in British finches in 2005 and 2006. The gross and histopathological findings are consistent with upper alimentary tract lesions caused by *T. gallinae* infection in columbiform species [24,25], although lesions in finches typically occur in the proximal

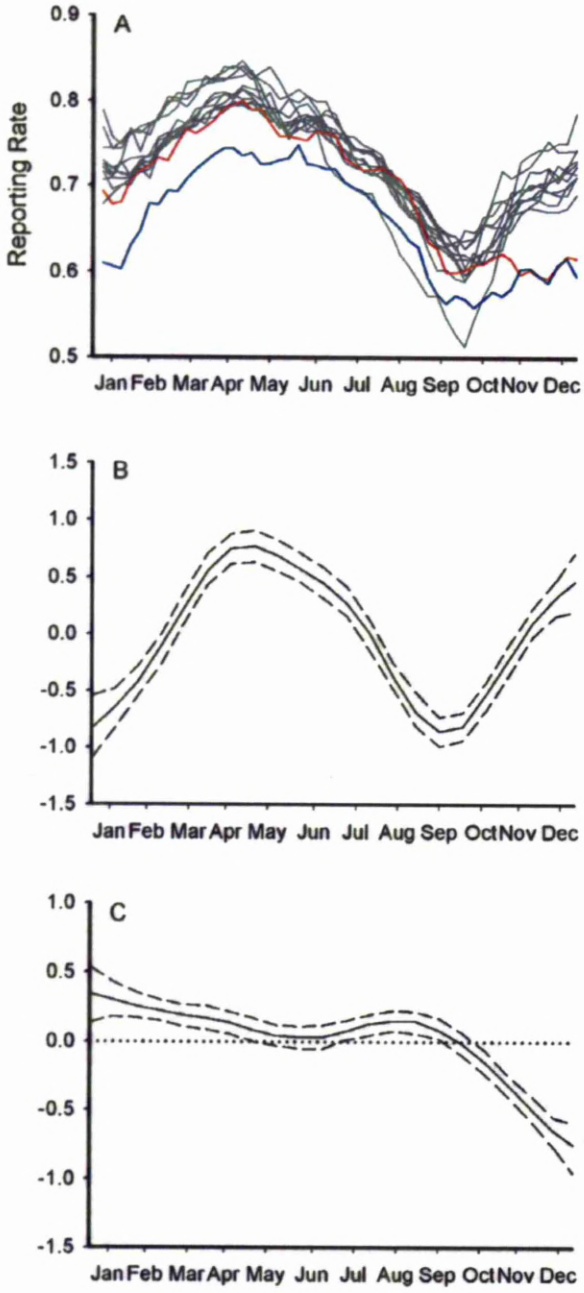

Figure 5. Seasonal variation in greenfinch occurrence in gardens. (a) Reporting rate for greenfinch in all GBW gardens for the years 1996–2005 (grey lines), 2006 (red) and 2007 (blue). (b) Fitted seasonal pattern of mean peak greenfinch count in 828 GBW gardens with complete counts in 2005. (c) Difference in mean peak count throughout the year between 2005 and 2006 for greenfinch, dashed lines represent 95% confidence limits.
doi:10.1371/journal.pone.0012215.g005

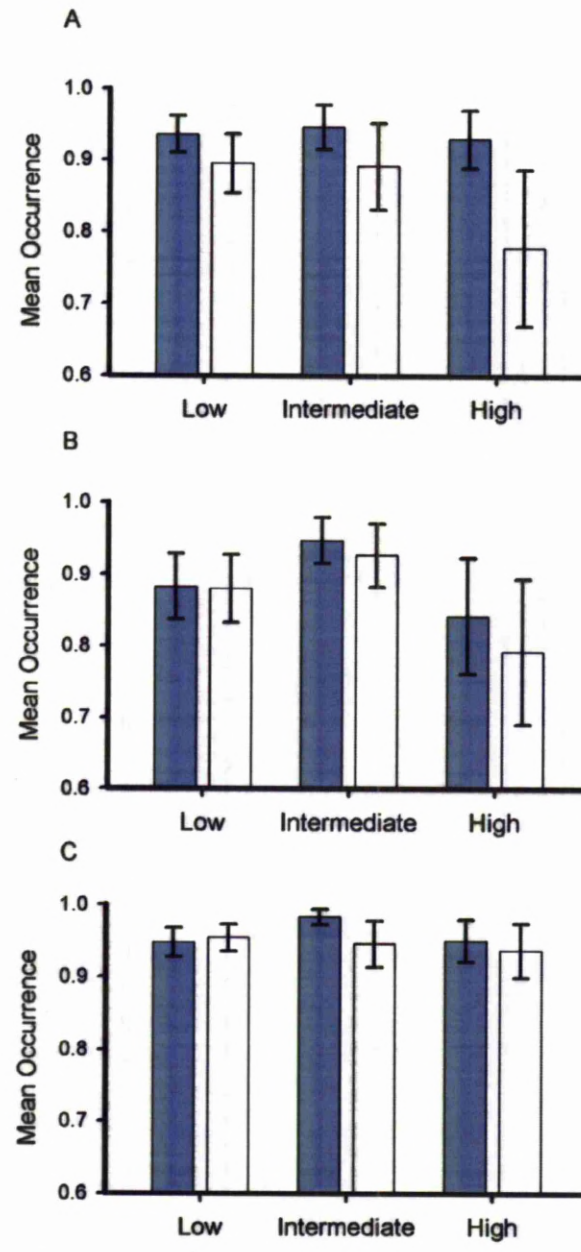

Figure 6. Regional change in greenfinch occurrence in gardens in response to trichomonosis. Mean reporting rate from GBW of greenfinch, chaffinch and dunnock in spring 2005/06 (filled bars) and 2007 (open bars) in areas of Low, Intermediate and High incidence of trichomonosis incidence (see Fig. 3). Bars represent 95% confidence limits.
doi:10.1371/journal.pone.0012215.g006

oesophagus, compared to the pharyngeal region in affected pigeons and doves. Ultrastructural examination identified morphology consistent with *Trichomonas* sp. protists, including *T. gallinae* [26–28]. Amplification and sequencing of the ITS1/5.8S/ITS2 region using TFR1 and TFR2 primers yielded consensus sequence with 100% identity to published accounts for *T. gallinae*, confirming the parasite species identification [10,29–31].

Experimental assessment of the survival of *T. gallinae* in white-winged dove *Zenaida asiatica* carcasses found that most reliable

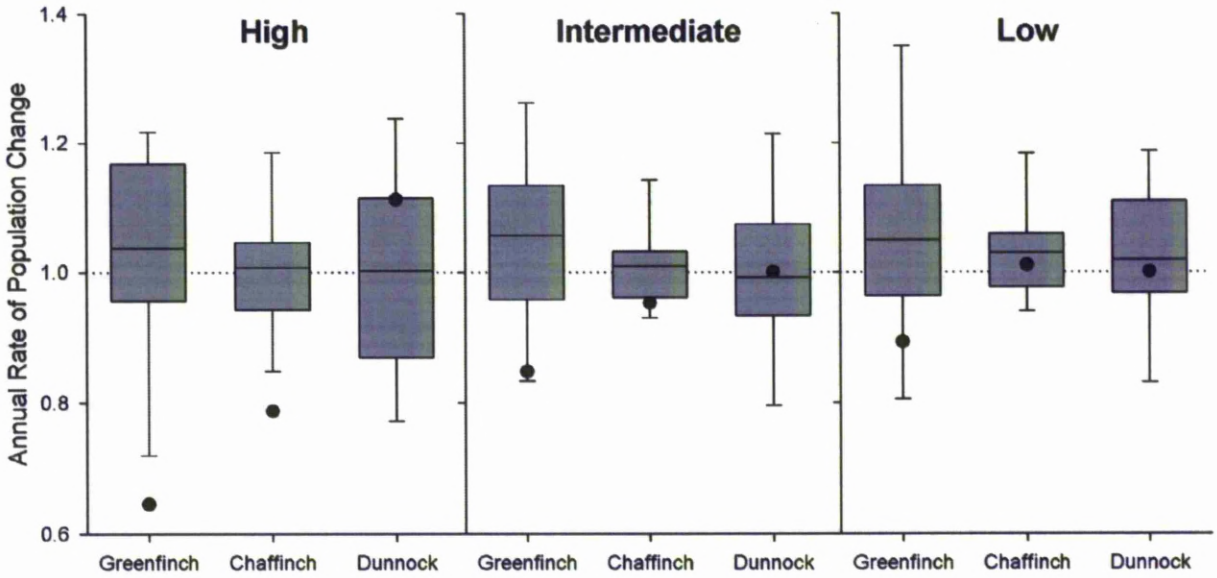

Figure 7. Annual rate of population change as measured by the BBS in areas of differing disease incidence. Boxes show mean and quartiles of annual changes and whiskers minimum and maximum annual change observed in the period 1994–2006; points, the population change recorded in 2007. Dotted line indicates no population change.
doi:10.1371/journal.pone.0012215.g007

diagnostic results were obtained on sampling within 8 hours of death and that parasite culture from *T. gallinae*-positive birds was successful in only 44% of carcasses sampled at 48 hours following death [32]. In the current study, parasite culture was found to be a useful technique for confirmation of the diagnosis in carcasses even in a mild state of autolysis. Due to delays in submission, however, negative culture results could not be used to exclude the diagnosis. Therefore, nested PCR provided a useful ancillary diagnostic tool in combination with post mortem and microbiological examinations within our case definition for the diagnosis of trichomonosis for carcasses in which the protozoan parasites were no longer viable.

Consideration of two independent data sets of bird mortality in Britain (i.e. (1) surveillance based opportunistically on unsolicited reports from members of the public and (2) systematic monitoring according to a defined protocol by our volunteer network) in combination enables us to maximise their relative benefits. The large-scale, but ad hoc, sampling from the opportunistic survey provided indication of the commencement of the epidemic and samples to determine its epidemiology. The systematic sampling using a pre-existing network provided a quantitative measure of incidence which will be relatively robust to, for example, increases in reporting frequency in response to media coverage. Such data could not realistically be collected in any other way and should, over time, provide much needed detail on the background complement of disease agents in wild bird populations.

The winter of 2006 was relatively mild (mean temperature anomaly in England for Nov-Mar +1.7°C [33]) so mortality levels would be expected to be low [34]. Also, the number of birds, especially seed-eaters, recorded in gardens would be expected to be lower than usual due to a reduced reliance on provisioning [35] and a small reduction in the reporting rate of dunnock was observed in the area of high disease incidence perhaps for this reason. However, the much larger reduction in the occurrence of greenfinches in gardens was not simply because birds were not coming into gardens for food, since reporting continued to be low into the following spring (Fig. 5) and regional declines in greenfinch breeding populations were observed in an independent dataset (BBS) with broader habitat coverage. The onset of the decline in greenfinch reporting rate is contemporaneous with the onset of the trichomonosis outbreak (Fig. 5) and the spatial pattern in regional population decline (Table 1) matches that of the disease

Table 1. Population change of breeding birds between 2006 and 2007.

	Low		Intermediate		High	
	n	Change	n	Change	n	Change
Greenfinch	477	−10.9 (−17.3, −4.1)	433	−15.2 (−21.8, −8.1)	232	−35.5 (−42.3, −27.9)
Chaffinch	549	+0.6 (−3.7, +5.1)	526	−4.4 (−8.7, +0.2)	256	−21.3 (−25.7, −16.5)
Dunnock	496	−1.2 (−7.0, +7.3)	465	−0.3 (−7.7, +7.6)	286	+11.4 (+1.7, +22.1)

Population change between 2006 and 2007 in areas of High, Intermediate and Low incidence of trichomonosis mortality during autumn 2006 derived from the Breeding Bird Survey. The percentage change between the two years is given with approximate 95% confidence limits, n is the number of BBS squares in each region in which the species was recorded.
doi:10.1371/journal.pone.0012215.t001

occurrence (Fig. 4). We have been unable to identify any other factor that could have caused such large-scale mortality. Furthermore, there were no similarly large declines noted in any other garden bird species (Toms, *unpublished data*).

In Great Britain as a whole, the greenfinch breeding population increased steadily (by c. 60%) from the mid 1980s to 2006, but decreased (significantly) by 15% in 2007 compared to the previous year [36]. It seems likely that this decline was driven, in large part, by the emergence of trichomonosis. Given that the population of greenfinches in Great Britain is in the order of 4 million [23], this represents mortality of a very large number of individuals, possibly in the order of half a million birds. This level of mortality due to an EID is unprecedented in British wild bird populations and is of international interest as, although other studies have demonstrated trichomonosis in non-columbiform species [10], this is the first trichomonosis epidemic to show substantial negative impact on free-ranging populations of these species. The current study also reinforces the value of large-scale, long-term, citizen science programmes for wildlife population monitoring and disease surveillance and of a multi-disciplinary approach to investigating the conservation significance of wildlife disease. As the opportunistic reports from the public were unsolicited, rather than in response to a direct appeal, such as the one undertaken to monitor the spread of house finch mycoplasmosis in the USA [20], we predict large-scale under-reporting of the 2006 trichomonosis epidemic. Our use of volunteers to actively search for sick and dead birds in a systematic fashion, however, should provide a reliable estimate of relative disease incidence (with up to 10% of monitored gardens recording at least one incident of morbidity or mortality) and this was temporo-spatially consistent with the observed population declines in greenfinch and chaffinch populations.

The origin of *T. gallinae* infection in finches is currently unknown but columbiform species are considered the most likely source. Future research priorities include molecular studies to identify the origin of *T. gallinae* in finches, for example through comparing parasite isolates derived from finch, columbiform and other wild bird species, and to assess *T. gallinae* strain diversity in Great Britain on the basis of geography, species and virulence. Retrospective surveys using the nested PCR methodology on archived garden bird tissues, collected prior to 2005, could identify presence of the parasite in non-columbiform populations prior to the onset of epidemic finch mortality, although the chance of detecting a low prevalence from the relatively small number of archived carcasses would be low.

Although greenfinches appear to be the species most frequently affected by *T. gallinae* infection in garden habitats (and chaffinches somewhat less so), the reasons for this are not clear. The greenfinch is one of the species most frequently affected by other infectious diseases that are commonly diagnosed in garden birds, such as salmonellosis and colibacillosis (Lawson and Cunningham, *unpublished data*). The gregarious and granivorous habits of finch species sharing food and water at feeding stations with high contact rates are likely to facilitate pathogen spread. Trichomonosis, however, was confirmed only rarely in members of the Paridae, which also commonly flock to garden bird feeders, so feeding behaviour is unlikely to be the sole driver of greenfinch susceptibility. Investigations of wildlife species mortality which rely on the reporting of sick and dead birds by members of the public have an inherent risk of bias due to variation in observer effort. Also, some species, such as large or brightly coloured ones, are more likely to be detected. Systematic sampling by volunteers in our network provides a relatively consistent level of observer effort and will have reduced variability in detection bias between gardens so should provide a robust measure of relative disease incidence. Although we have quantified the occurrence of trichomonosis in dead birds, the overall prevalence of *T. gallinae* infection in wild bird populations remains unknown. Prospective studies to screen multiple species of live birds for trichomonad parasites [37] would help address this knowledge gap. Experimental challenge studies in multiple species are required to definitively confirm the extent of interspecific variation in susceptibility to infection with, and to disease caused by, *T. gallinae*.

Land-use change and habitat degradation have led to an increased national focus on garden habitats as a useful refuge for British wildlife [38]. It has been estimated that 48% of gardens in Britain provide some form of artificial food for wildlife [39]. Anthropogenic provisioning of wild birds in garden habitats influences contact rates among conspecifics and alters species complements at feeding sites; both factors influence pathogen transmission and exposure rates [40]. Garden bird feeding practice in Great Britain has altered over recent years with increased adoption of summer feeding and increased provision of sunflower and niger seed which might have led to increased concentration of birds at feeders. *Trichomonas gallinae* can be transmitted through direct contact between birds, for example courtship and feeding of young, and through indirect routes including shared food and water sources [9,41], however further studies are required to assess the relative importance of these transmission routes for finches. Establishing the nature and frequency of disease transmission at garden feeders is thus clearly important to identify if mitigation measures are required and, if so, how they should be employed. The greenfinch and chaffinch are both common garden bird visitors in England and Wales across the year, ranking number 9 and 10 in the most frequent garden visitors in the GBW scheme [22]. Both species are gregarious, visiting gardens in flocks, but other granivorous passerine species are reported in a comparable number of gardens around feeding stations, for example house sparrows, great tits and blue tits, and they also feed in groups. More generally, *T. gallinae* is a pathogen of potential significance to the racing pigeon, aviculture, game bird and poultry industries and the implications for finch trichomonosis to these industries remain poorly understood. Continued monitoring of diseases in wild bird populations is required to better quantify and understand their impact on population dynamics [6,42] and to identify future changes in host-parasite relationships.

Materials and Methods

Ethics statement

No live animals were used for this research, however, the project was reviewed and approved by the Zoological Society of London's Ethics Committee

Identification of the disease epidemic

Since 2000, opportunistic nationwide monitoring of the causes of garden bird mortality in Great Britain has been carried out by the Institute of Zoology (IoZ, London), the Wildlife Veterinary Investigation Laboratory (Cornwall) and the Scottish Agricultural Colleges (Ayrshire). In 2005, these organisations, together with the BTO, the RSPB, the Department of Veterinary Pathology, University of Liverpool and the Universities Federation for Animal Welfare, established a coordinated surveillance network as part of the Garden Bird Health initiative (GBHi). The GBHi was established before the emergence of trichomonosis in British finches was identified and has always included wide-ranging investigations to identify pathogens responsible for garden bird disease.

The GBHi surveillance of garden bird morbidity and mortality takes place via opportunistic reports obtained from the general

public and weekly reports from identified volunteers who form a systematic reporting network. As opportunistic reports are vulnerable to temporal or spatial observer bias, for example following regional media reports, the systematic surveillance network was established in April 2005 in order to quantify disease prevalence rates. This network utilised the BTO's Garden BirdWatch (GBW) volunteer network of approximately 15,000 households throughout Britain [22]. A random sample of 1,614 volunteers (stratified by the number of participants in each recording region) were approached with a view to recording additional information relating to the incidence of diseased and dying birds in their gardens. Of those GBW volunteers approached, 752 submitted data to the GBHi project in 2006. A visual examination of the spatial distribution of sites suggested no spatial bias in those who submitted data relative to those initially approached and there was no difference in the distribution of latitudes ($F1,2356 = 1.14$, NS) or longitudes ($F1,2356 = 1.31$, NS) between the two samples.

Identification of disease organism

Post mortem examinations using a standardised protocol were performed on carcasses submitted from a subset of reported garden bird mortality incidents. Cases were selected for post-mortem investigation based on fresh carcass availability. Birds thought to have died as a result of trauma, predation or infectious disease were examined; our selection criteria did not specifically or solely target finch species or suspected cases of trichomonosis, but rather aimed to achieve a representative cross-section of species and aetiologies. In 2005 and 2006, a combined total of 995 garden birds of 42 species were examined, of which Fringillidae species accounted for 64% of submissions.

Fresh carcasses were submitted by post or were hand-delivered and were refrigerated at 4°C and examined fresh within 48 hours of submission where possible, or were frozen at −20°C on submission and examined at a later date. Each submitted carcass was assigned a unique post mortem reference code and the species, age, sex and body weight were recorded for each bird examined. Systematic external and internal examinations of body systems were performed and any gross lesions described. Where indicated, and where the state of carcass decomposition permitted, samples were taken for microbiological, parasitological and histopathological investigations.

Liver and contents of the mid-small intestinal loop were sampled aseptically from the majority of cases, as were any lesions found, and were examined for the presence of pathogenic bacteria. Briefly, liver was plated directly onto the following media: Colombia blood agar supplemented with 5% horse or sheep blood (CBA)(QCM laboratories, UK or E and O laboratories, UK), incubated under aerobic conditions, and Chocolate blood agar (CCBA) (QCM laboratories), incubated under 5–10% CO_2 conditions. Small intestinal contents were plated directly onto the following media: (1) Xylose-Lysine Deoxycholate (XLD) agar (QCM laboratories), or MacConkey agar without salt (E&O laboratories) and Brilliant green agar (E&O laboratories), incubated under aerobic conditions; (2) CBA, incubated under aerobic conditions; (3) Campylobacter Blood Free Selective medium (modified CCDA-Preston) (QCM laboratories), incubated under microaerophilic conditions, and (4) immersed into selenite Salmonella-selective enrichment broth (QCM laboratories or E&O laboratories) under aerobic conditions for 24 h followed by subculture onto XLD agar aerobically. At the Institute of Zoology, liver also was plated onto CBA and incubated anaerobically. The same bacteriology protocol was used for examination of necrotic ingluvitis lesions as for the intestinal contents with the exception of the modified CCDA-Preston media. Bacterial isolates were identified using colony and Gram's staining morphology, followed by biochemical properties which were determined using the API biochemical test strip method (API-BioMerieux, Marcy l'Etoile, France).

In addition, oesophageal lesions (circa 5 mm^3) from cases with necrotic ingluvitis were incubated at 30°C in Trichomonas Media No. 2. (Oxoid, UK) and screened for motile trichomonads at 24, 48, 72 hrs and 5 days. Wet mount preparations of small intestinal contents were examined in a subset of cases for evidence of nematode, cestode and protozoan parasites.

Samples from a range of organs (including brain, gizzard, heart, kidney, liver, lung, pectoral muscle, small intestine, spleen, trachea and any diseased tissues), were fixed in neutral-buffered 10% formalin and processed for histopathological examination using routine methods. Duplicate samples of organs and diseased tissues were stored frozen at −20°C or −80°C for future analyses.

A combination of morphological and molecular techniques was used to identify the trichomonad species. Giemsa-stained preparations of trichomonad cultures were examined using light microscopy to assess parasite morphology. These were prepared by placing a drop of active trichomonas culture onto a standard glass microscope slide; this was then air dried, alcohol-fixed and stained using routine methods. Transmission and scanning electron microscopy was performed on trichomonad cultures fixed in 2.5% buffered gluteraldehyde and post-fixed in 1% osmium tetroxide (VWR, UK) at the University College Medical School, Royal Free Campus, using Philips 201 and 501 microscopes.

DNA was extracted from frozen/thawed necrotic ingluvitis lesions collected from finches using the Biosprint 15 DNA Blood Kit (Qiagen, UK) for purification of DNA from tissue according to the manufacturer's instructions. DNA was extracted from trichomonad cultures using the same technique. PCR was used to amplify the ITS1/5.8S/ITS2 ribosomal region using published TFR1 and TFR2 primers [30] with an adapted protocol. Briefly, PCR reactions were run with 3 µL of 10X PCR buffer (Qiagen), 3 µL of 25 mM $MgCl_2$ (Qiagen), 0.5 µL of 5 U/µL HotStar Taq Plus DNA Polymerase (Qiagen), 2 µL template DNA, 0.4 µL of 100 mM dNTP mix (Bioline, UK), 3 µL of 10 µM forward and reverse primer and molecular grade water to complete the 50 µL per reaction. Oligonucleotide primers were supplied by Operon Biotechnologies, Germany. After an initial 15 min denaturation at 94°C, 35 cycles of 94°C for 1 min, 65°C for 30 sec and 72°C for 1 min were carried out, followed by a 5 min extension at 72°C using a thermal cycler (Tec-571, Techne, UK). Each PCR run contained a negative control of water and a positive control of purified trichomonad DNA obtained from parasites cultured from an affected greenfinch found dead as part of this study.

The PCR products, consisting of a clear single band, were visualised under UV light after ethidium bromide staining of a 1% agarose gel and the expected product size (circa 400 bps) was confirmed using Ready-Load 100 bp DNA ladder (Invitrogen, UK). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and submitted for sequencing at the John Innes Genome Laboratory, UK, using the Applied Biosystems 3730xl with POP7 polymer and the TFR1 and TFR2 primers. Chromatograph profiles were inspected using Chromas 2 software (www.synthesisgene.com). Sequences from the forward TFR1 primer and the reverse complement of the TFR2 primer PCR product were aligned in both directions for each sample using MEGA 4.1 software and ClustalW (www.megasoftware.net). Sequences were compared with available gene sequences within NCBI Genbank using the BLAST search function to determine species identification within the Trichomonadidae.

Molecular detection of Trichomonas infection

A nested PCR protocol was designed to increase the sensitivity of detection of trichomonad parasite DNA in template DNA extracted from lesions sampled post mortem and to provide a diagnostic tool for cases where autolysed carcass condition precluded *Trichomonas* sp. culture. Trichomonad small subunit (SSU) rRNA primers (forward -TACTTGGTTGATCCTGCC and reverse - TCACCTACCGTTACCTTG) from [43] were used for the first reaction. The PCR product nucleotide sequence was obtained from a pure trichomonad culture obtained from an affected greenfinch. Nested primers, TN3 forward (ATAGGACTGCAAAGCCGAGA) and TN4 reverse (TGATTTCACCGAGTCATCCA), were then designed using Prime3 online software [44]. Primers were supplied by Eurofins MWG Operon (UK).

The first stage used 2 μL of 10X PCR buffer (Qiagen), 0.1 μL of 5 U/μL HotStar Taq Plus DNA Polymerase (Qiagen), 2 μL template DNA, 0.4 μL of 10 mM each dNTP mix (Qiagen), 0.4 μL of 100 μM forward and reverse primer and molecular grade water to complete the 20 μL reaction. After an initial 5 min denaturation at 94°C, 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 mins were carried out, followed by a 5 min extension at 72°C using a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, UK).

The reaction mixture for the second amplification was the same as for the first, except for the use of the nested primers. The amplification mix comprised 19 μl of mix and 1 μl of PCR product template from the first amplification round. After an initial 5 min denaturation at 94°C, 35 cycles of 94°C for 45 sec, 50°C for 45 sec and 72°C for 45 sec were carried out, followed by a 7 min extension at 72°C. Each amplification contained a negative control, consisting of water and a positive control of purified DNA obtained from cultured trichomonad parasites from a greenfinch. Amplified PCR products were visualised under UV light after ethidium bromide staining of a 3% agarose gel and the expected product size (circa 200 bps) was confirmed using Easy Ladder I (Bioline). PCR products were submitted for sequencing at Cogenics (UK) using the ABI 3730 xl platform with the TN3 forward and TN4 reverse primers. Chromatograph profiles were inspected using Chromas 2 software. The sequence from the forward TN3 primer and the reverse complement of the TN4 primer PCR product were aligned in both directions for each sample using MEGA 4.1 software and ClustalW. Sequences were compared with available gene sequences within NCBI Genbank using the BLAST search function.

Reliable discrimination between necrotic ingluvitis due to salmonellosis or trichomonosis in finches is not possible based on gross examination alone. In order to evaluate whether our nested PCR cross-reacted non-specifically with *Salmonella* Typhimurium Definitive Type (DT)40 and DT56 variant (v), lesions from 56 greenfinches and six chaffinches confirmed to have died due to salmonellosis by microbiological examination in 2005 and 2006 (Lawson, *unpublished data*) were screened. The majority of cases were negative on nested PCR (54/62 cases) and all cases examined during the study period 1 April 2006 to 30 September 2006 were negative. This indicates that 8 cases examined outside the study period might have had concurrent infection with *Trichomonas* sp. and *Salmonella* sp.. Carcass condition precluded parasite culture in all but one of these cases, but this case yielded trichomonad parasites confirming the existence of dual infection. These findings suggest that the nested PCR does not cross react non-specifically with *Salmonella* Typhimurium DT40 and DT56(v) and can be used within our case definition for the diagnosis of trichomonosis in finches.

Case definition

Cases of trichomonosis were diagnosed on the basis of the presence of necrotic ingluvitis lesions in combination with positive culture of motile trichomonads and/or positive nested PCR amplification. Salmonellosis also causes necrotic ingluvitis and this was diagnosed by lesions being positive for *Salmonella* sp. on culture. No cases of dual infection were identified during the study period.

Geographical and temporal distribution of the epidemic

Garden Bird Watch participants (c. 9000) record the presence of a range of bird and other wildlife species encountered each week throughout the year; a subset (c. 30%) submit actual counts of each species seen using an online recording form. Participants maintain a consistent level of observational effort from one week to the next; data from weeks that are under- or over-observed are discarded. Variation in observer effort and competence is inevitable, but this can be controlled for by introducing a site effect into the models used to examine the data [40]. Almost all the participants provide food of some kind (the range of food provided for garden birds is also recorded on a weekly basis) for wild birds and feeding stations are generally the focal point of the study areas. The subset of participants that took part in GBH*i* surveyed all or part of their garden systematically in a consistent manner each week to record the number, and putative cause, of dead or sick birds found. The clinical signs of ill health in birds affected by trichomonosis typically included non-specific malaise, although dysphagia was noted in a large proportion of reported incidents. This contrasts with the clearly recognisable external signs of conjunctivitis caused by *Mycoplasma gallisepticum* in the house finch and which were used to monitor spatial spread of mycoplasmosis [20].

In order to evaluate the geographical distribution of the trichomonosis epidemic, data from the opportunistic and systematic reporting schemes were examined for the period between 1 April and 30 September 2006. This interval was selected to minimise the likelihood of confounding these data with mortality due to salmonellosis, outbreaks of which occur during the winter months and which also result in non-specific signs of malaise in finches. Previous studies of salmonellosis in Great Britain [45], continental Europe [46] and North America [47] along with our own observations on salmonellosis in passerines over the period 1995–2008 (Lawson and Cunningham, *unpublished data*), have shown the disease to be seasonal, occurring almost exclusively within the period 1st October to 31st March.

Incident definition

As resources and logistics did not allow all dead birds found to be submitted for post mortem examination, we established specific criteria for determining if a mortality incident should be classified as likely being due to trichomonosis. For the purposes of this analysis, a trichomonosis incident was defined if, within the six month period of the study (between 1 April and 30 September 2006), mortality included two or more dead finches (greenfinch or chaffinch), one or more sick finch(es) with typical signs of disease, or if trichomonosis was confirmed post mortem. To give a measure of incidence for the opportunistic reports, we expressed the total number of trichomonosis incidents reported in each county per thousand households according to the 2001 UK National Census [48].

Changes in bird abundance

Each week, GBW participants recorded the presence of each bird species in their garden and, optionally, the peak number of

birds counted each week [22]. For this analysis we used the 828 gardens for which data were submitted in every week from January 2005 to June 2007 in England south of 53.5°N (see below). We modelled bird abundance as the proportion of gardens in which the species was present ('reporting rate'). We modelled reporting rates using generalised additive or linear mixed models, fitted using the gamm function in package *mgcv* for R 2.6.0 [49,50].

To exclude the possibility that changes in other environmental factors, such as climate, between years might have caused changes in greenfinch numbers, we also modelled reporting rates of chaffinch, which has a similar body size and ecology to the greenfinch, and was the second most frequently affected species in which trichomonosis was recorded, and of dunnock, which also feeds around garden feeders [51] but in which trichomonosis was rarely recorded (only three cases diagnosed in 2005 and 2006 combined). Both species show similar patterns of spatial and temporal abundance in gardens to the greenfinch [22]. We predicted that, if trichomonosis was responsible for changes in numbers, chaffinch should show an identifiable, but less marked, response and dunnock should show little response; if other factors, such as climatic factors or a change in resource availability, were involved then the response among the three species should be similar.

Abundance of birds in gardens varies seasonally in a non-linear fashion, with peak numbers occurring in late winter or spring and lowest counts in late summer/autumn, when many birds are moulting [22,35]. To model this variation we fitted a generalised additive model to the reporting rate as a function of week number (1–7 Jan = 1 to 25–31 Dec = 52) in the form of a thin-plate regression spline using gamm with a binomial error structure and logit link function [50]. We used the default level of smoothing, as increasing the potential for smoothing (by changing the basis, k) did not alter the results materially. To account for variation among gardens in the probability of birds being present and observer ability we included garden identifier as a random-effect term. Preliminary models including garden identifier as a fixed effect showed the distribution of these effects, as for other large-scale schemes run by BTO, were indeed approximately normally distributed. The weekly records of bird presence are likely to exhibit serial auto-correlation, both temporally and spatially. We initially fitted models with no temporal or spatial auto-correlation and while temporal correlation among the residuals was strong, spatial correlations (analysed using the correlog function in library ncf [52]), though statistically significant, were typically weak ($r \leq 0.05$). This may reflect the fact that distance between gardens was generally much greater than the ambit of individual foraging flocks and it is unlikely the provisioning behaviour in any one sample garden influenced the behaviour in neighbouring sample gardens. We therefore included an AR1 auto-regressive correlation structure (with weeks numbered consecutively through the entire period) to account for the correlation between records in consecutive weeks; the degree of auto-correlation was assumed to be the same for each garden.

To quantify impacts on the breeding season following the epidemic of trichomonosis in autumn 2006, we constructed a model to compare reporting rate at the start of the 2007 breeding season (weeks 13–21, 26 March–28 May) with the average reporting rate for the same period in the previous two years (which, as far as could be determined, were typical). We restricted the analysis to the previous two years to avoid potential confounding effects of long-term trends in bird numbers and reporting rates. As we were considering a relatively short time-span within a year, it was not necessary to fit a smoothed term of week; rather we modelled reporting rate as a function of garden size, week (and its square to account for any non-linearity) and a two-level dummy variable, year, with weeks in 2005/06 having year = 0 and those in 2007 year = 1. The estimate of this latter term then gives the change in reporting rate in 2007 relative to that in the previous two years. As before, we included garden identifier as a random effect and fitted an auto-correlated binomial error structure.

Mortality due to trichomonosis varied spatially throughout the country, so we defined three regions representing areas of High, Intermediate and Low incidence of trichomonosis, based on the results of opportunistic sampling. We restricted these analyses to England south of a line from the Mersey to the Humber (approx. 53° 30′N) as it is in this region that gardens participating in GBW are most representative of the landscape as a whole (for example upland areas, where there tend to be few GBW sites, are relatively limited in extent). Greenfinch, chaffinch and dunnock populations occur widely across this region, being observed in 70–80% of gardens in the GBW scheme. For convenience, we defined these three regions in terms of administrative county boundaries with areas of High (Cheshire, Derbyshire, Gloucestershire, Herefordshire, Leicestershire, Shropshire, Staffordshire, Warwickshire and the West Midlands), Intermediate (Bedfordshire, Buckinghamshire Cambridgeshire, Lincolnshire, Northamptonshire, Nottinghamshire, Oxfordshire, South Yorkshire and Wiltshire) and Low (Berkshire, Essex, Hampshire, Hertfordshire, Greater London, Kent, Norfolk, Suffolk, Surrey and Sussex) incidence of trichomonosis. To test for differences in the change in reporting rate in spring 2007 among areas, we included an interaction term between area and the dummy year variable described above.

Numbers of birds present across Britain during the breeding season are monitored using line-transect counts in a sample of c. 3,000 randomly selected 1×1 km squares by the Breeding Bird Survey (BBS) [23]. BBS transects are undertaken in all habitats, rather than being restricted to gardens as with the GBW scheme. An index of relative abundance based on a generalized linear Poisson model with categorical site and year fixed-effects is produced annually; we obtained indices for the three county groupings to measure the relative change in breeding population between 2006 and 2007 in each region.

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Author Contributions

Conceived and designed the experiments: RR BL JKK AAC. Performed the experiments: BL MPT KMP JC IC ADE LAH SKJ TWP MWP PRR VRS. Analyzed the data: RR. Contributed reagents/materials/analysis tools: OCH KT. Wrote the paper: RR BL AAC. Data collection: BL MPT KMP JC IC LAH SKJ TWP MWP PRR VRS. Modelling of data: RR. Research design and coordination: JKK AAC. Supervision of RSPB data collection: ADE. Development of new molecular analytic tools: OCH. Technical assistance: MWP. Supervision of molecular analyses: KT.

References

1. Daszak P, Cunningham AA, Hyatt AD (2000) Emerging infectious diseases of wildlife- threats to biodiversity and human health. Science 287: 443–449.

2. Friend M, McLean RG, Dein FJ (2001) Disease emergence in birds: challenges for the twenty-first century. Auk 118: 290–303.

3. Bunbury N, Jones CG, Greenwood AG, Bell DJ (2008) Epidemiology and conservation implications of *T. gallinae* infection in the endangered Mauritian pink pigeon. Biol Cons 141: 153–161.
4. Sheldon BC, Verhulst S (1996) Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. Trends Ecol Evol 11: 317–321.
5. Reed TE, Daunt F, Hall ME, Phillips RA, Wanless S, et al. (2008) Parasite treatment affects maternal investment in sons. Science 321: 1681–1682.
6. Hochachka WM, Dhondt AA (2000) Density-dependent decline of host abundance resulting from a new infectious disease. Proc Natl Acad Sci USA 97: 5303–5306.
7. LaDeau SL, Kilpatrick AM, Marra PP (2007) West Nile virus emergence and large-scale declines of North American bird populations. Nature 447: 710–713.
8. Van Riper C III, Van Riper SG, Goff ML, Laird M (1986) The epizootiology and ecological significance of malaria in Hawaiian (USA) land birds. Ecol Monogr 56: 327–344.
9. Forrester DJ, Foster GW (2008) Trichomonosis. In: Atkinson CT, Thomas NJ, Hunter DB, eds. Parasitic diseases of wild birds. Iowa: Wiley-Blackwell. pp 120–153.
10. Anderson NL, Grahn RA, Van Hoosear K, Bondurant RH (2009) Studies of trichomonad protozoa in free ranging songbirds: Prevalence of *Trichomonas gallinae* in house finches (*Carpodacus mexicanus*) and corvids and a novel trichomonad in mockingbirds (*Mimus polyglottos*). Vet Parasitol 161: 178–186.
11. National Wildlife Health Center (2002) USGS National Wildlife Health Center Quarterly Wildlife Mortality Report October 2002 to December 2002. Available: http://www.nwhc.usgs.gov/publications/quarterly_reports/2002_qtr_4.jsp. (Accessed January 13, 2010).
12. Stabler RM (1954) *Trichomonas gallinae*. a review. Exp Parasitol 3: 368–402.
13. Höfle U, Blanco J, Palma L, Melo P (2000) Trichomoniasis in Bonelli's eagle (*Hieraaetus fasciatus*) nestlings in south-west Portugal. In: Lumej JT, Remple JD, Redig PT, Lierz M, Cooper JE, eds. Raptor Biomedicine III Zoological Education. Lake Worth, FL: Network Inc. pp 4–52.
14. Real J, Mañosa S, Muñoz E (2000) Trichomoniasis in a Bonelli's eagle population in Spain. J Wildl Dis 36: 64–70.
15. Pennycott TW, Park A, Mather HA (2006) Isolation of different serovars of *Salmonella enterica* from wild birds in Great Britain between 1995 and 2003. Vet Rec 158: 817–820.
16. Holmes P, Duff P (2005) Ingluvitis and oesophagitis in wild finches. Vet Rec 157: 455.
17. Pennycott TW, Lawson B, Cunningham AA, Simpson V, Chantrey J (2005) Necrotic ingluvitis in wild finches. Vet Rec 157: 360.
18. Eidson M, Komar N, Sorhage F, Nelson R, Talbot T, et al. (2001) Crow deaths as a sentinel surveillance system for West Nile virus in the northeastern United States, 1999. Emerg Infect Dis 7: 615–620.
19. Chvala S, Bakonyi T, Bukovsky C, Meistera T, Bruggerd K, et al. (2007) Monitoring of Usutu virus activity and spread by using dead bird surveillance in Austria, 2003–2005. Vet Microbiol 122: 237–245.
20. Dhondt AA, Tessaglia DL, Slothower RL (1998) Epidemic mycoplasmal conjunctivitis in house finches from eastern North America. J Wildl Dis 34: 265–280.
21. Altizer S, Hochachka WM, Dhondt AA (2004) Seasonal dynamics of mycoplasmal conjunctivitis in eastern North American house finches. J Anim Ecol 73: 309–322.
22. Cannon AR, Chamberlain DE, Toms MP, Hatchwell BJ, Gaston KJ (2005) Trends in the use of private gardens by wild birds in Great Britain 1995-2002. J Appl Ecol 42: 659–671.
23. Newson SE, Evans KL, Noble DG, Greenwood JJD, Gaston KJ (2008) Use of distance sampling to improve estimates of national population sizes for common and widespread breeding birds in the UK. J Appl Ecol 45: 1330–1338.
24. Perez-Mesa C, Stabler RM, Berthrong M (1961) Histopathological changes in the domestic pigeon infected with *Trichomonas gallinae* (Jones-Barn strain). Avian Dis 5: 48–60.
25. Narcisi EM, Sevolan M, Honigberg BN (1991) Pathologic changes in pigeons infected with a virulent *Trichomonas gallinae* strain (Eiberg). Avian Dis 35: 55–61.
26. Benchimol M (2004) Trichomonads under Microscopy. Microsc Microanal 10: 528–550.
27. Soulsby EJL. In:, Helminths, Arthropods and Protozoa of Domesticated Animals. London: Balliere, Tindall and Cassell. pp 583–586.
28. Bondurant RH, Honigberg BM (1994) Trichomonads of veterinary importance. In: Kreier JP, ed. Parasitic Protozoa. New York: Academic Press. pp 111–206.
29. Kleina P, Bettim-Bandinelli J, Bonatto SL, Benchimol M, Bogo MR (2004) Molecular phylogeny of Trichomonadidae family inferred from ITS-1, 5.8S rRNA and ITS-2 sequences. Int J Parasitol 34: 963–970.
30. Gaspar da Silva D, Barton E, Bunbury N, Lunness P, Bell DJ, et al. (2007) Molecular identity and heterogeneity of Trichomonad parasites in a closed avian population. Infect Genet Evol 7: 433–40.
31. Gerhold RW, Yabsley MJ, Smith AJ, Ostergaard E, Mannan W, et al. (2008) Molecular characterization of the *Trichomonas gallinae* morphologic complex in the United States. J Parasitol 94: 1335–41.
32. Erwin KG, Kloss C, Lyles J, Felderhoff J, Fedynich AM, et al. (2000) Survival of *Trichomonas gallinae* in white-winged dove carcasses. J Wildl Dis 36: 551–554.
33. Met Office (2010) Met Office: UK Actual and Anomaly maps. Available: Http://www.metoffice.gov.uk/climate/uk/anomalygraphs/index.html. (Accessed January 13 2010).
34. Robinson RA, Baillie SR, Crick HQP (2007) Weather-dependent survival: implications of climate change for passerine population processes. Ibis 149: 357–364.
35. Chamberlain DE, Vickery JA, Glue DE, Robinson RA, Conway GJ, et al. (2005) Annual and seasonal trends in the use of garden feeders by birds in winter. Ibis 147: 563–575.
36. Risely K, Noble DG, Baillie SR (2008) The Breeding Bird Survey 2007, BTO Research Report 508. Thetford: British Trust for Ornithology. 20 p.
37. Bunbury N, Bell D, Jones C, Greenwood A, Hunter P (2005) Comparison of the InPouch TF Culture System and Wet-Mount Microscopy for Diagnosis of *Trichomonas gallinae* Infections in the Pink Pigeon *Columba mayeri*. J Clin Microbiol 43: 1005–1006.
38. Toms MP (2007) Are gardens good for birds or birdwatchers? British Wildlife 19: 77–83.
39. Davies ZG, Fuller RA, Loram A, Irvine KN, Sims V, et al. (2009) A national scale inventory of resource provision for biodiversity within domestic gardens. Biol Cons 142: 761–771.
40. Robb GM, McDonald RA, Chamberlain DE, Bearhop S (2008) Food for thought: supplementary feeding as a driver of ecological change in avian populations. Front Ecol Environ 6: 476–484.
41. Bunbury N, Jones CG, Greenwood AG, Bell DJ (2007) *Trichomonas gallinae* in Mauritian columbids: implications for an endangered endemic. J Wildl Dis 43: 399–407.
42. Hudson PJ, Dobson AP (1989) Population biology of *Trichostrongylus tenuis*, a parasite of economic importance for red grouse management. Parasitol Today 5: 283–91.
43. Cepicka I, Kutisova K, Tachezy J, Kulda J, Flegr J (2005) Cryptic species within the *Tetratrichomonas gallinarum* species complex revealed by molecular polymorphism. Vet Parasitol 128: 11–21.
44. Rozen S, Skaletsky HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S, eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology. Totowa, NJ: Humana Press. pp 365–386.
45. Pennycott TW, Cinderey RN, Park A, Mather HA, Foster G (2002) *Salmonella enterica* subspecies *enterica* serotype Typhimurium and *Escherichia coli* 086 in wild birds at two garden sites in south-west Scotland. Vet Rec 151: 563–567.
46. Refsum T, Handeland K, Baggesen DL, Holstad G, Kapperud G (2002) Salmonellae in avian wildlife in Norway from 1969 to 2000. Appl Environ Microbiol 68: 5595–5599.
47. Hall AJ, Saito EK (2008) Avian wildlife mortality events due to Salmonellosis in the United States. J Wildl Dis 44: 585–593.
48. Census 2001 (2002) 2001 Census in England and Wales. Available: http://www.statistics.gov.uk/census2001/census2001.asp. (Accessed January 13 2010).
49. R Development Core Team (2007) R: A Language and Environment for Statistical Computing. Vienna, Austria. Available: http://www.R-project.org.
50. Wood SN (2006) Generalized additive models: An introduction with R. London: Chapman and Hall. 392 p.
51. Snow DW, Perrins CM (1998) The birds of the western Palearctic, concise edition; Vol. 2. Oxford: Oxford University Press. 1694 p.
52. Bjornstad ON, Ims RA, Lambin X (1999) Spatial population dynamics: analyzing patterns and processes of population synchrony. Trends Ecol Evol 14: 427–432.

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Acute necrotising pneumonitis associated with *Suttonella ornithocola* infection in tits (Paridae)

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ABSTRACT

Suttonella ornithocola, first isolated from the lungs of British tit species in 1996, was found to be a novel bacterium belonging to the family *Cardiobacteriaceae*. Comprehensive surveillance of garden bird mortality across Great Britain between April 2005 and April 2009 involved post mortem and microbiological examination of 82 tits (Paridae; multiple species) and six long-tailed tits (Aegithalidae; *Aegithalos caudatus*). *S. ornithocola* was isolated from six birds submitted from six incidents of morbidity and mortality involving Paridae and Aegithalidae species with a wide geographical distribution. The mortality incidents occurred sporadically at low incidence throughout the study period, which suggested that the infection is endemic in native bird populations, with a seasonal peak during early spring. Histopathological examination showed multiple foci of acute pulmonary necrosis associated with Gram-negative cocco-bacillary bacteria. These findings supported the hypothesis that *S. ornithocola* is a primary pathogen of tits in Great Britain.

Introduction

A variety of infectious diseases has been reported to cause morbidity and mortality of garden birds in Great Britain and continental Europe. The pathogens can be (1) bacterial, such as *Salmonella* Typhimurium (**Pennycott et al., 1998**; **Refsum et al., 2003**) and *Escherichia coli* serotype O86 (**Foster et al., 1998**), (2) viral, for example, avian pox (**Weli et al., 2004**) and (3) protozoal (e.g., *Trichomonas gallinae*; **Lawson et al., 2006**). Finches (greenfinch *Carduelis chloris*, chaffinch *Fringilla coelebs* and siskin *Carduelis spinus*) and house sparrows *Passer domesticus* are the species in which mortality incidents have most frequently been documented in Great Britain (**Pennycott et al., 1998, 2006**), whilst reports of infectious disease in tit species (Paridae and Aegithalidae) are comparatively rare (**Kirkwood et al., 2006**).

Members of the Paridae, and to a lesser extent the Aegithalidae, are common birds in garden habitats in Great Britain, particularly those with feeding stations (**Toms, 2003**), and their breeding populations are widely distributed across the country (**Risely et al., 2008**). **Kirkwood et al. (2006)** reported investigations into the morbidity and mortality of members of the Paridae and Aegithalidae at 11 disparate gardens in the spring of 1996; the species involved comprised the blue tit (*Cyanistes caeruleus*), coal tit (*Periparus ater*), great tit (*Parus major*) and long-tailed tit (*Aegithalos caudatus*). A total of 34 dead birds were reported, ranging from 1 to 10 per incident. Post mortem examinations revealed pulmonary congestion, but no other significant abnormalities. The bacterium, *Suttonella ornithocola*, was isolated from the lungs of affected birds (**Kirkwood et al., 2006**) and was identified as a novel bacterium belonging to the family *Cardiobacteriaceae* (**Foster et al., 2005**). **Kirkwood et al. (2006)** postulated that *S. ornithocola* infection might have caused the deaths of these birds, but could reach no firm conclusion as to the association between *S. ornithocola* and disease or mortality.

In this article, we describe six further mortality incidents affecting Paridae and Aegithalidae species that occurred between April 2005 and April 2009, from which *S. ornithocola* was isolated from lung tissue.

Material and methods

A national surveillance programme (the Garden Bird Health initiative (GBHi)) was launched in April 2005 to investigate causes of mortality in garden birds across Great Britain. The GBHi utilised a combination of two independent and complementary reporting schemes: (1) the opportunistic reports of garden bird morbidity and mortality solicited from the general public, and (2) systematic surveillance via the

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British Trust for Ornithology's (BTO) Garden Bird Watch (GBW)[1] volunteer network. Birds found dead in gardens were selected for post mortem investigation based on fresh carcass availability and were submitted by post. Details of the date found, geographical location and clinical signs observed were recorded.

Post mortem examinations were performed using a standardised protocol by a regional network of disease investigation laboratories comprising: the Institute of Zoology (London, England); the Department of Veterinary Pathology, University of Liverpool (Wirral, England); the Wildlife Veterinary Investigation Centre (Cornwall, England), and the Scottish Agricultural College (Ayrshire, Scotland). The age, sex and bodyweight (BW) were recorded for each bird examined. Birds were classed as juveniles until the post-juvenile body moult was complete. First year birds beyond their post-juvenile moult and adult birds were not differentiated; all were classified as adult for the purpose of this study. Sex was assigned based on gonadal inspection and/or plumage characteristics. The state of carcass preservation was described in each case as mild, moderate or advanced state of autolysis. Systematic external and internal examinations of body systems were performed and any gross lesions described. A subjective measure of body condition (emaciated, thin, moderate or good) was made based on visual inspection of the pectoral muscle and fat deposits.

Lung, liver and/or contents from the mid small intestinal loop were sampled aseptically from the majority of carcasses and examined for the presence of pathogenic bacteria. Briefly, lung and liver were plated directly onto each of the following media: Colombia blood agar supplemented with 5% horse or sheep blood (CBA; QCM laboratories or E and O laboratories); incubated under aerobic conditions, and Chocolate blood agar (CCBA; QCM laboratories); incubated under 5–10% CO_2 conditions.

Small-intestinal contents were plated directly onto each of the following media: (1) xylose–lysine deoxycholate (XLD) agar (QCM laboratories), or MacConkey agar without salt (E and O laboratories) and Brilliant green agar (E and O laboratories), incubated under aerobic conditions; (2) CBA, incubated under aerobic conditions; (3) Campylobacter blood free selective medium (modified CCDA-Preston; QCM laboratories), incubated under microaerophilic conditions; and (4) immersed into selenite Salmonella-selective enrichment broth (QCM laboratories or E and O laboratories) under aerobic conditions for 24 h followed by subculture onto XLD agar aerobically.

At the Institute of Zoology, liver also was plated onto CBA and incubated anaerobically. All culture media were incubated at 37 °C, with the exception of selenite broth at the Scottish Agricultural College, which was incubated at 42 °C. Heart blood was examined using the same protocol as the liver.

Suttonella ornithocola isolates were identified by their morphology (Gram-negative rod to coccobacillus, 0.5–0.6 × 0.6–1.3 μm), preference for aerobic and capnophilic (or carboxyphilic) culture conditions, oxidase positive reaction and biochemical test results (API 20NE, BioMérieux), as described by **Kirkwood et al. (2006)**. 16S rRNA genes were amplified by PCR using universal primers pA and sequenced to confirm the isolate identity as *S. ornithocola* (**Foster et al., 2005**).

Where the state of carcass preservation permitted, samples from a range of organs (including any lesions found) were fixed in neutral-buffered 10% formalin and were processed for histopathological examination using routine methods. Tissue sections were stained using haematoxylin and eosin or Gram-Twort stains.

Results

A total of 82 members of the family Paridae (30 blue tit *Cyanistes caeruleus*, 11 coal tit *Periparus ater*, 40 great tit *Parus major*, six marsh tit *Parus palustris*) and six members of the family Aegithalidae (all long-tailed tits *Aegithalos caudatus*) were examined post mortem between April 2005 and April 2009. The age distribution comprised 69 adults, 14 juveniles, and five birds for which the age category was undetermined.

Bacteriology was performed on at least one tissue from each bird carcass submitted for post mortem examination, except for one bird where the level of decomposition precluded meaningful examination. Bacterial culture of the lung was performed on 61% (54/88) of the birds examined, which included 80% (20/25) of those diagnosed as having died of infectious disease, 63% (17/27) of those diagnosed as having died due to trauma other than predation, 56% (15/27) of those for which the cause of death was undetermined, 29% (2/7) of those diagnosed as having died from predation, and 50% (1/2) of those diagnosed as having died from 'other' causes.

S. ornithocola was isolated from the lungs of six tits considered to have died as a result of infectious disease (**Table 1**); no evidence of the bacterium was found in the tits which died from other causes. Liver was cultured from 72% (63/88) of birds examined; small-intestinal contents were cultured from 88% (77/88) of birds examined and heart blood was cultured from two birds. *S. ornithocola* was isolated from the liver of three birds and from the heart blood of a single bird, all of which were also positive for the bacterium on lung culture (**Table 2**).

All *S. ornithocola* isolates had morphological and culture characteristics typical of those reported previously for this bacterium (**Foster et al., 2005; Kirkwood et al., 2006**). For each isolate, 16S rRNA sequence data was identical to that of the type strain (Genbank Accession Number AJ717394).

Birds were submitted from mortality incidents throughout Great Britain and *S. ornithocola* infection was found in England, Scotland and Wales (**Fig. 1**). Details of *S. ornithocola*-positive mortality incidents are presented in **Table 1**. Several individuals of multiple species were seen to be affected in 5/6 incidents. Mortality or morbidity of blue tits was reported in each incident. Where sick birds were observed, they exhibited non-specific signs of malaise, such as lethargy and fluffed-up plumage (**Table 1**). No evidence of contemporaneous morbidity or mortality was observed in other garden bird species at affected sites. All incidents occurred in the spring, between the end of January and the end of April, and all were observed around garden feeding stations.

A summary of the details of the confirmed cases of *S. ornithocola* infection is presented in **Table 2**. All cases were in fully-fledged birds that had hatched prior to the calendar year of death. Typically, poor facial plumage condition was observed with peri-oral matting of feathers and there usually was urate staining around the vent. In each case, the upper alimentary tract was empty and the gizzard contained only dark-stained grit, indicating that the bird had not recently fed. Pulmonary congestion was noted in four cases, whilst the respiratory tract appeared grossly normal in the remaining two birds. No other significant abnormalities were noted. Microscopic examinations of wet smear preparations of small-intestinal contents were performed in 4/6 cases and all were negative for metazoan or protozoan parasites.

Histopathological examination was performed on the lung from 4/6 birds from which *S. ornithocola* was cultured and from multiple tissues from two of these birds. A list of the tissues examined is presented in **Table 2**. Multiple foci of acute pulmonary necrosis associated with clusters of Gram-negative rods were present in three birds (**Fig. 2**). A mixture of Gram-positive and Gram-negative rods was present in some of the air spaces and surrounding tissue in the fourth bird (Incident 2), but there was no evidence of tissue necrosis. The lungs of two birds (Incidents 2 and 5) were markedly congested and a small number of parabronchi in these birds contained acellular eosinophilic material (oedema fluid). Large mononuclear cells with foamy cytoplasm (macrophages) and lymphocytes were present within, and lining, air spaces in three birds (Incidents 2, 5 and 6). It is possible that some of the foamy cells lining the air spaces were reactive type II pneumocytes, but no additional investigations were conducted to investigate this further. No abnormalities were detected in the other organs examined.

Of the 82 tits (Paridae) and six long-tailed tits examined post mortem between April 2005 and April 2009, infectious disease was determined to be the sole cause of death, or an important contributory factor to the cause of death, in 25 (28%) birds. *S. ornithocola* was considered to be the primary pathogen in six (7%) birds, avian pox in eight (9%), trichomonosis in five (6%) and miscellaneous pathogens in the remaining six birds. Of the non-infectious causes of death, seven (8%) tits died of predation, 27 (31%) of other trauma and two (2%) of other causes (gizzard impaction).

The cause of death was undetermined for 27 birds as the more autolysed the carcass, the less chance there was of reaching a diagnosis. The cause of death was undetermined for 4/15 (27%) cases in

[1] See: **http://www.bto.org/gbw**.

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Table 1
Details of mortality incidents from which birds were submitted and *Suttonella ornithocola* was isolated, April 2005–April 2009. The total number of birds found dead per incident is given. The total number of birds seen sick per incident is given in parentheses.

Incident reference number	Species affected number found dead (seen sick)	Month and year	County	Clinical signs
Incident 1	Blue tit 6(0) Coal tit 0 (1)	April 2005	Cambridgeshire, England	Lethargic
Incident 2	Blue tit 1(3) Great tit 1(0)	April 2006	Kent, England	Fluffed up and lethargic
Incident 3	Blue tit 1(1) Coal tit 0(1) Long-tailed tit 0(1)	April 2007	Monmouthshire, Wales	Fluffed up
Incident 4	Blue tit 1(1)[a]	April 2007	Essex, England	Fluffed up and lethargic
Incident 5	Blue tit 1(0) Long-tailed tit 1(0)	January 2009	Tayside, Scotland	No sick birds observed
Incident 6	Blue tit 2(1) Coal tit 2(0)	April 2009	Tayside, Scotland	Fluffed up

[a] May have been multiple birds affected; only one sick blue tit seen in garden at any one time over a period of 3 weeks.

Table 2
Summary of the tit cases from which *Suttonella ornithocola* was isolated.

Incident reference number	Species	Age	Sex	Bodyweight (g)	Body condition	Lung congestion present?	Tissues cultured for bacteria	State of carcass preservation	Histopathology
Incident 1	Blue tit	Adult	Male	9.9	Good	No	Heart blood, liver, small intestine (SI), lung[a]	Mild autolysis	Heart, kidney liver, lung
Incident 2	Blue tit	Adult	Male	7.9	Thin	No	Liver,[a] SI, lung[a]	Moderate autolysis	Brain, heart, kidney, liver, lung, pectoral muscle, trachea
Incident 3	Blue tit	Adult	Male	8.3	Thin	Yes	Liver, SI, lung[a]	Moderate autolysis	NA
Incident 4	Blue tit	Adult	Male	8.0	Thin	Yes	SI, lung[a]	Moderate autolysis	NA
Incident 5	Blue tit	Adult	Female	8.0	Thin	Yes	Liver,[a] SI, lung[a]	Moderate autolysis	Lung only
Incident 6	Coal tit	Adult	Male	8.0	Moderate	Yes	Liver,[a] SI, lung,[a] heart[a]	Moderate autolysis	Lung only

[a] Positive for *Suttonella ornithocola*.

a mild state of autolysis; 17/59 (29%) cases in a moderate state of autolysis; and 6/14 (43%) cases in an advanced state of autolysis. *S. ornithocola* has fastidious conditions for growth and the likelihood of successful culture might have been reduced in the autolysed samples. Consequently, *S. ornithocola* infection might be under-reported, particularly from cases with a poor state of carcass preservation.

Discussion

In this study, *S. ornithocola* infection was diagnosed from six incidents of mortality involving members of the Paridae between April 2005 and April 2009. Two of these incidents also involved morbidity or mortality of long-tailed tits (Aegithalidae). Prior to this study, incidents of tit mortality associated with *S. ornithocola* infection had only been demonstrated in Great Britain in 1996 (**Kirkwood et al., 2006**) and, to this date, not reported elsewhere.

Infected birds usually were in thin body condition and, in all cases, the appearance of the alimentary tract suggested that they had been anorexic prior to death. Poor plumage condition was noted in each case, which suggested that the bird had not been preening adequately for a period prior to death, consistent with the observed clinical signs of non-specific malaise. **Kirkwood et al. (2006)** also reported clinical signs as being non-specific, including fluffed-up plumage, lethargy and reduced awareness. In addition, in separate incidents, these authors reported gaping in birds, as if they were trying to swallow, or as showing excessive thirst, but such signs were not observed in the current study.

On post mortem examination, gross congestion of the lungs was present in 4/6 birds examined and this observation was made in 4/21 birds examined by **Kirkwood et al. (2006)**. Histopathological examination demonstrated acute necrotising pneumonitis associated with Gram-negative rods, morphologically consistent with *S. ornithocola*, in 3/4 birds for which the lung was examined microscopically. Pulmonary congestion and/or oedema were evident in three birds, including the one without necrotic lesions. No abnormalities were found in the other tissues after histological examination.

S. ornithocola was cultured from the lung in each infected tit and from the liver or heart blood in 3/6 cases. No hepatic lesions were detected on gross or histopathological examination. Post mortem bacterial overgrowth might explain culture of the bacterium from multiple sites in these tits, although disseminated infection and bacteraemia could have occurred before death. No evidence of *S. ornithocola* infection was found in tits that died of trauma or predation during the study period. The results support the hypothesis that *S. ornithocola* is a causative agent of pulmonary disease in tits. The relative importance of *S. ornithocola* infection as a cause of morbidity and mortality in Paridae and Aegithalidae species, in comparison with that due to other pathogens and non-infectious causes (such as predation or starvation) is unknown, but the absolute number of mortality incidents confirmed in this study suggested that disease outbreaks caused by this bacterium occurred at a low incidence.

S. ornithocola-associated mortality incidents had a seasonal distribution, between late January and April, which is consistent with the temporal cluster of cases reported by **Kirkwood et al. (2006)**,

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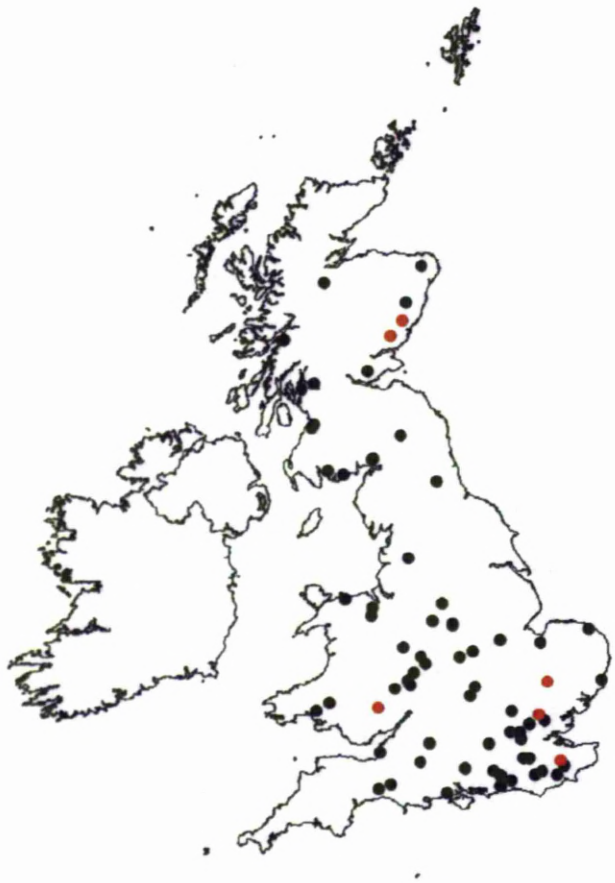

Fig. 1. Distribution of the mortality incidents from which tit carcasses were examined during the study period, April 2005–April 2009. Red circles indicate the distribution of mortality incidents from which *Suttonella ornithocola* was isolated. Black dots indicate the distribution of other mortality incidents from which tit carcasses were examined.

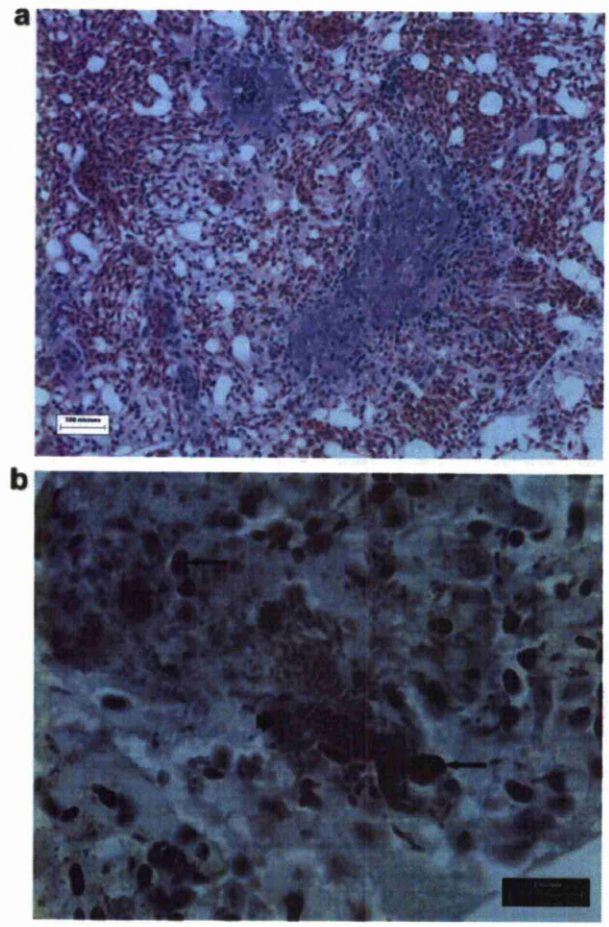

Fig. 2. Acute necrotising bacterial pneumonitis in a blue tit (Incident 1): (A) Haematoxylin and eosin; (B) Gram-Twort. Note the colony of Gram-negative rods (arrowhead) and small number of mononuclear inflammatory cells (arrows).

which all occurred between March and May. **Kirkwood et al. (2006)** hypothesised that this apparent seasonality occurs because the pathogen is spread in late spring and early summer, possibly facilitated through the arrival of summer migrants. In the spring months, increased contact rates between birds might occur, for example associated with breeding activity.

In the current study, *S. ornithocola* infection was diagnosed only in birds beyond their post-juvenile moult (categorised as adult) although it was not possible to differentiate between first year and adult birds on the basis of our plumage records. Future research should clearly discriminate whether birds are typically affected by *S. ornithocola* infection in their first year of life or also in subsequent years. Five of the six birds with *S. ornithocola* infection in this study were male. A similar skewed sex ratio was seen in 1996, where 14/16 birds examined were male, although the reason for this, or even if it represents a true trend, is not known. It is plausible that gender-related behavioural differences during the breeding season might explain the bias toward males observed so far. **Kirkwood et al. (2006)** noted that exposure to a common risk factor also might be involved and that all affected birds were in provisioned gardens, as was the case in the current study.

We identified mortality incidents associated with *S. ornithocola* infection in England, Scotland and Wales, indicating that this bacterium is geographically widespread in Great Britain. Similarly, there was no evidence of geographical clustering in the incidents reported by **Kirkwood et al. (2006)**, which occurred across 10 counties in England and Wales.

As with 10/11 incidents reported by **Kirkwood et al. (2006)**, mortality of blue tits was reported in all *S. ornithocola* incidents in this study, although concurrent morbidity and/or mortality of other Paridae or Aegithalidae species were reported in all but one of the incidents (**Table 1**). This might suggest that blue tits play a role in the epidemiology of the infection, or are particularly susceptible to *S. ornithocola* infection.

The current study has shown multiple, sporadic incidents of tit mortality associated with *S. ornithocola* infection over a 4 year period. This pattern suggested that *S. ornithocola* infection might be endemic within native tit populations and contrasts with the findings in 1996 where a temporal cluster of 11 incidents was seen over a period of 3 months. The mechanism of transmission of *S. ornithocola* is currently unknown, but as the infection is associated with acute necrotising pneumonitis, aerosol transmission would be a likely route of spread.

Conclusions

Further investigations are required to extend our understanding of the epidemiology and significance of *S. ornithocola* infection in birds. Our study suggested that this bacterium is an endemic, primary pathogen of tit species in Great Britain, causing disease outbreaks at a low incidence across the country with a seasonal

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(spring) trend. Continued monitoring to increase the number of confirmed incidents will help to determine whether the gender skew towards males and the apparent predisposition of the blue tit to disease found in this study, and in a previous study by Kirkwood et al. (2006), are true.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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References

Foster, G., Ross, H.M., Pennycott, T.W., Hopkins, G.F., McLaren, I.M., 1998. Isolation of Escherichia coli O86:K61 producing cyto-lethal distending toxin from wild birds of the finch family. Letters in Applied Microbiology 26, 395–398.

Foster, G., Malnick, H., Lawson, P.A., Kirkwood, J., Macgregor, S.K., Collins, M.D., 2005. *Suttonella ornithocola* sp. nov., from birds of the tit families, and emended description of the genus *Suttonella*. International Journal of Systematic and Evolutionary Microbiology 55, 2269–2272.

Kirkwood, J.K., Macgregor, S.K., Malnick, H., Foster, G., 2006. Unusual mortality incidents in tit species (family Paridae) associated with the novel bacterium *Suttonella ornithocola*. Veterinary Record 158, 203–205.

Lawson, B., Cunningham, A., Chantrey, J., Hughes, L., Kirkwood, J., Pennycott, T., Simpson, V., 2006. Epidemic finch mortality. Veterinary Record 159, 367.

Pennycott, T.W., Ross, H.M., McLaren, I.M., Park, A., Hopkins, G.F., Foster, G., 1998. Causes of death of wild birds of the family Fringillidae in Britain. Veterinary Record 143, 155–158.

Pennycott, T.W., Park, A., Mather, H.A., 2006. Isolation of different serovars of *Salmonella enterica* from wild birds in Great Britain between 1995 and 2003. Veterinary Record 158, 817–820.

Refsum, T., Vikøren, T., Handeland, K., Kapperud, G., Holstad, G., 2003. Epidemiologic and pathologic aspects of *Salmonella typhimurium* infection in passerine birds in Norway. Journal of Wildlife Diseases 39, 64–72.

Risely, K., Noble, D.G., Baillie, S.R., 2008. The Breeding Bird Survey 2007, BTO Research Report 508. British Trust for Ornithology, Thetford, pp. 1–20.

Toms, M., 2003. The BTO/CJ Garden BirdWatch Book. British Trust for Ornithology, Thetford, UK, pp. 1–128.

Weli, S.C., Okeke, M.I., Tryland, M., Nilssen, O., Traavik, T., 2004. Characterization of avipoxviruses from wild birds in Norway. Canadian Journal of Veterinary Research 68, 140–145.

[2] See: http://www.birdcare.org.uk.
[3] See: http://www.bva-awf.org.uk.
[4] See: http://www.birdfood.co.uk/.
[5] See: http://www.cranswickpetproducts.co.uk/.
[6] See: http://www.gardman.co.uk/.
[7] See: http://www.rspb.org.uk/.
[8] See: http://www.ufaw.org.uk.

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